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CARBON SOURCE UTILIZATION AND ISOENZYME ANALYSIS AS TAXONOMIC AIDS FOR TOXIGENIC *NEOSARTORYA* SPECIES AND THEIR RELATIVES

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Collection strains representing species belonging to the genus *Neosartorya* and its relatives from section *Fumigati* of the genus *Aspergillus* were compared for some of their phenotypic features. The examination of both the carbon source utilization and isoenzyme patterns provided a useful tool for clustering these strains. Many species (e.g. *Neosartorya hiratsukae*, *N. quadricincta*, *N. spinosa*, *N. aurata*, *N. aureola*) could readily be distinguished from other species based on their specific isoenzyme and carbon source utilization spectra. Close relationship was observed between the *A. fumigatus* and *N. fischeri* strains. *Aspergillus* strain FRR 1266, which also revealed distinct mitochondrial DNA and nuclear DNA patterns, and amplified DNA profiles, was the closest relative of the recently described *N. pseudofischeri* species, and could also be distinguished from the *A. fumigatus* strains by its specific carbon source utilization patterns. High levels of variability were detected among *N. glabra* and *A. viridinutans* strains; most of the strains of Australian origin formed distinct clusters.

The genus *Neosartorya* (family *Trichocomaceae*) was established by Malloch and Cain [1] to accommodate teleomorphs of species belonging in the "*Aspergillus fischeri* series" of the *A. fumigatus* species group [2]. The anamorphs of these species, and their relatives lacking a sexual stage belong to section *Fumigati* of the genus *Aspergillus* [3]. This section now involves 12 sexual *Neosartorya* species, and 5 asexual *Aspergilli* [4]. The most important species among them is *Aspergillus fumigatus* Fresenius, which is a ubiquitous filamentous fungus in the environment, and also an important human pathogen

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[2]. Other species of this section are also regarded as possible agents of fungal infections; e.g. *N. pseudofischeri* strains have been encountered from human lesions [5], and *N. fennelliae* strains have been isolated from the eyeballs of a rabbit [6]. *N. fischeri* isolates have frequently been reported as heat resistant spoilage fungi in foods [7]. In addition, various mycotoxins are produced by these species, many of which may cause serious health hazard [8, 9]. Some species also have valuable properties for the fermentation industry; e.g. one *N. spinosa* strain has been used for the production of optically active 1,3-butanediol (US patent no. 05326705), while *A. fumigatus* strains are used for the production of fumagillin, which exhibits amebicidal activity [10].

Several taxonomic studies have been carried out recently on *A. fumigatus*, and other species belonging in section *Fumigati*. Among other approaches, secondary metabolite profiles and micromorphology of the strains were examined [8, 9, 11], DNA reassociation kinetics of some of the species were recorded [5], exocellular polysaccharides of the strains were characterized [12], protein and antigen profiles were analyzed [13], and isoenzyme and amplified DNA patterns of some species were compared [14]. Our aim was to evaluate the applicability of carbon source utilization patterns and isoenzyme analysis for the taxonomic examination of species in section *Fumigati*. Substrate utilization spectra have proved to be very useful for yeasts, where carbon source assimilation and fermentation tests are frequently used for species delimitations [15]. Isoenzyme analysis was successfully applied in the taxonomy of sections *Ornati*, *Cremeri* and *Flavi* of the *Aspergillus* genus [16, 17].

Materials and methods

Strains. The strains examined are listed in Table I. Strains were maintained on malt extract agar slants. For protein extractions, the strains were grown in a liquid minimal medium [18] at 30 °C for 48 h on a rotary shaker.

Isoenzyme analysis. Crude protein extracts were prepared as described previously [19]. Polyacrylamide slab gel electrophoresis was carried out according to Davis [20]. Three percent (w/v) stacking gels and 7.5% (w/v) separation gels were used. After a 10-min prerun, the samples were loaded and run at a constant voltage of 200 V overnight. A Pharmacia GE-4 vertical gel slab electrophoretic apparatus was used with four 3 mm slabs. Isoenzyme activities were detected as detailed earlier [14]. β -Arylesterase (EC 3.1.1.2), acid phosphatase (EC 3.1.3.1), NADP-dependent glutamate dehydrogenase (EC 1.4.1.4), superoxide dismutase (EC 1.15.1.1), and NADP-dependent malate dehydrogenase (EC 1.1.1.38) activities were recorded.

Carbon source utilization tests. About 80 compounds were tested as sole carbon sources in our experiments. Each compound was analyzed at concentrations of 0.2% in a minimal medium (MM; 1% KH_2PO_4 , 0.5% $(\text{NH}_4)_2\text{SO}_4$, 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% Wickerham's vitamin solution) [21]. Appropriate amounts of hot minimal medium were poured into sterile flasks containing the required amounts of the compounds to be tested. After mixing well, equal volumes were poured into Petri dishes. The pH of the medium was always between 5 and 6. Plates were inoculated with conidial suspensions (approximately 10^7 ml^{-1}) of the strains, and incubated at 30 °C in the dark. Growth intensities were compared after incubation for four and seven days with a control plate not containing any carbon source.

Statistical analysis. The statistical analysis of the data was carried out by using the SYN-TAX-pc version 5.0 software package [22]. The binomial matrix obtained from the carbon source utilization

and isoenzyme data was used to calculate the Jaccard coefficients [23]. The coefficient matrices were analyzed by an unweighted pair-group method using arithmetic averages (UPGMA) [23]. All calculations were performed on an IBM-compatible AT computer.

Results and discussion

The isoenzyme patterns of the strains were highly polymorphic. The least polymorphic patterns were observed in the cases of glutamate dehydrogenase and superoxide dismutase isoenzymes, while β -arylesterase and acid phosphatase patterns permitted differentiation between individual isolates of the same species. Similar results were obtained in our earlier studies concerning the isoenzyme patterns of *A. fumigatus* strains [14]. Mating type strains of the heterothallic *Neosartorya fennelliae* and *N. spathulata* species could not be distinguished by isoenzyme analysis, with the exception of the β -arylesterase patterns of *N. fennelliae* NRRL 5534 and NRRL 5535 strains [14]. As an example, the β -arylesterase isoenzyme patterns of some strains are shown in Fig. 1.

Table I

The Neosartorya and Aspergillus strains examined [type or netotype strains are labelled with (T), while strains of Australian origin are typed in boldface]

No. Strain	Strain number, origin, remarks
1 <i>Aspergillus brevipes</i>	(T) NRRL 2439, ATCC 16899 (from soil, Australia)
2 <i>Aspergillus duricaulis</i>	IMI 217288 (from cereal, Iraq)
3 <i>Aspergillus duricaulis</i>	(T) NRRL 4021, (from soil, Argentina)
4 <i>Aspergillus fumigatus</i>	(T) NRRL 163; (from chicken lung Connecticut, USA)
5 <i>Aspergillus fumigatus</i> mut. <i>helvola</i>	(T) NRRL 174 (light coloured spontaneous mutant)
6 <i>Aspergillus fumigatus</i> var. <i>acolumnaris</i>	(T) NRRL 5587; IMI 174456 (from soil, India)
7 <i>Aspergillus fumigatus</i> var. <i>ellipticus</i>	(T) NRRL 5109 (from the lungs of a patient, USA)
8 " <i>Aspergillus fumigatus</i> "	FRR 1266 (from soil, Warrumbungle Mtns, Australia)
9 <i>Aspergillus unilateralis</i>	IMI 062876 (from rhizosphere of <i>Epacris impressa</i>, Australia)
10 <i>Aspergillus unilateralis</i>	NRRL 577 (origin unknown)
11 <i>Aspergillus viridinutans</i>	IMI 133982 (from soil, Moscow, USSR)
12 <i>Aspergillus viridinutans</i>	IMI 182127 (from Pinus caribea, Sri Lanka)
13 <i>Aspergillus viridinutans</i>	IMI 280490 (from soil, Zambia)
14 <i>Aspergillus viridinutans</i>	IMI 306135 (from soil, W. Australia)
15 <i>Aspergillus viridinutans</i>	(T) IMI 062875 (=NRRL 4365; from rabbit dung, Australia)
16 <i>Aspergillus viridinutans</i>	(T) NRRL 576 (=NRRL, 4365; from rabbit dung, Australia)

Continued on p. 4.

Table I continued

17	<i>Aspergillus viridinutans</i>	NRRL 6106 (origin unknown)
18	<i>Neosartorya aurata</i>	(T) NRRL 4379, ATCC 16894 (from jungle soil, Brunei)
19	<i>Neosartorya aurata</i>	NRRL 4379 (from jungle soil, Brunei)
20	<i>Neosartorya aureola</i>	NRRL 2391 (from soil, Liberia)
21	<i>Neosartorya aureola</i>	(T) NRRL 2244, ATCC 16896 (from soil, Gold Coast, Ghana)
22	<i>Neosartorya fennelliae</i>	NHL 2951 (from marine sludge, Japan; mating type A)
23	<i>Neosartorya fennelliae</i>	NHL 2952 (from marine sludge, Japan; mating type a)
24	<i>Neosartorya fennelliae</i>	(T) NRRL 5534 (from eye ball of a rabbit, USA; mating type A)
25	<i>Neosartorya fennelliae</i>	(T) NRRL 5535 (from eye ball of a rabbit, USA; mating type a)
26	<i>Neosartorya fischeri</i>	NRRL A-7223 (from soil, Peoria, Illinois, USA)
27	<i>Neosartorya fischeri</i>	(T) NRRL 181, ATCC 1020 (from canned apples)
28	<i>Neosartorya glabra</i>	IMI 131700 (from man's ear swab, Australia)
29	<i>Neosartorya glabra</i>	(T) IMI 061447 (from rubber tyre scrap, Iowa, USA)
30	<i>Neosartorya glabra</i>	IMI 061450 (from garden soil, Australia)
31	<i>Neosartorya glabra</i>	NRRL 183, Thorn #5047 (from beer wort, Lyon, France)
32	<i>Neosartorya glabra</i>	(T) NRRL 2163 (from rubber tyre scrap, Iowa, USA)
33	<i>Neosartorya hiratsukae</i>	(T) NHL 3008; CBS 294.93 (from aloe juice, Japan)
34	<i>Neosartorya hiratsukae</i>	NHL 3009; CBS 304.93 (from indoor air, Japan)
35	<i>Neosartorya pseudofischeri</i>	(T) NRRL 20748 (from human vertebrae, Atlanta, USA)
36	<i>Neosartorya pseudofischeri</i>	(T) NRRL 3496 (from moldy cardboard, Canada)
37	<i>Neosartorya quadricincta</i>	IMI 058374 (from soil, Australia)
38	<i>Neosartorya quadricincta</i>	(T) NRRL 2154, ATCC 16897 (from cardboard, UK)
39	<i>Neosartorya quadricincta</i>	NRRL 2221 (from fabric, Florida, USA)
40	<i>Neosartorya</i> sp.	NRRL 4179 (from soil, Australia " <i>N. glabra</i> ")
41	<i>Neosartorya spathulata</i>	(T) NHL 2948; CBS 408.89 (from soil, Taiwan; mating type A)
42	<i>Neosartorya spathulata</i>	(T) NHL 2949; CBS 409.89 (from soil, Taiwan; mating type a)
43	<i>Neosartorya spinosa</i>	NRRL 3435, NHL 5084 (from Y. Sato, Japan)
44	<i>Neosartorya spinosa</i>	(T) NRRL 5034, ATCC 18698 (from soil, Nicaragua)
45	<i>Neosartorya straminea</i>	(T) NRRL 4652, ATCC 16895 (from soil, Wisconsin, USA)
46	<i>Aspergillus cervinus</i>	IMI 107684 (from soil in a tropical rain forest, Malaysia)

Substrate utilization patterns of the strains were also variable. Some carbon sources were utilized by all strains examined, while some others were not appropriate as sole source of carbon for any of the strains. Results for the compounds tested are as follows:

Compounds utilized by all strains: D-mannose, D-arabinose, D-xylose, melibiose, raffinose, D-glucose, D-mannitol, cellobiose, sucrose, D-ribose, gluconic acid, β -keto-D-gluconic acid, fumaric acid, L-malic acid, succinic acid, pyruvic acid, acetic acid, L-proline, L-phenylalanine, starch.

Compounds not utilized by any of the strains: methanol, cytosine, cytidine, uracil, orotic acid, DL-isocitric acid, L-leucine, L-methionine, L-cysteine, L-histidine, D-tryptophan.

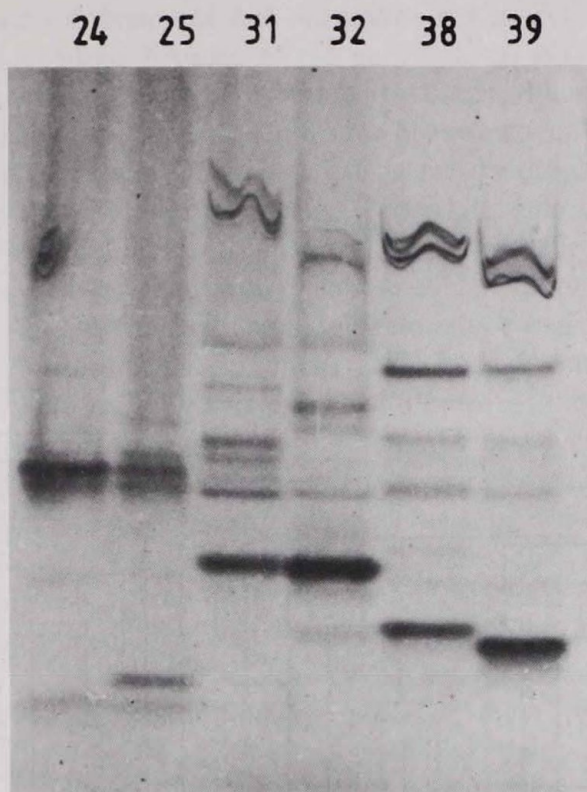


Fig. 1. β -Arylesterase isoenzyme patterns of some strains representing species of section *Fumigati* of the *Aspergillus* genus. Strain numbers are those listed in Table I

Compounds utilized by all strains except for *A. cervinus* IMI 107684: L-alanine, L-asparagine, L-glutamic acid, L-glutamine, L-ornithine, L-arginine, ascorbic acid, inositol, ethanol, glycerol, β -amino-butyric acid.

Compounds utilized variably: D-lyxose, L-rhamnose, L-sorbose, melezitose, lactose, glucosamine, erythritol, xylitol, galactitol, adonitol, salicin, L-tyrosine, L-valine, L-serine, L-lysine, L-threonine, glycine, glycylglycine, L-tryptophan, L-citrulline, cis-aconitic acid, α -ketoglutaric acid, vanillic acid, polygalacturonic acid.

A dendrogram generated by using an UPGMA clustering of the Jaccard coefficients of the similarity matrix obtained from the results of the isoenzyme analysis and carbon source utilization patterns is shown in Fig. 2. Altogether, 132 isoenzyme bands, and 67 carbon source utilization patterns were taken into account. An interesting finding is the distant relationship of some Australian isolates to other strains representing the same species based on their isoenzyme and carbon source utilization patterns. One example is, the distant relationship observed between *N. glabra* IMI 061450 (strain 30 in Fig. 2) and IMI 131700 (strain 28 in Fig. 2), to the other *N. glabra* strains. The second is the great evolutionary distance observed between *A. viridinutans* IMI 306135 (strain 14), *A. viridinutans* IMI 062875 (strain 15) and NRRL 576 (strain 16) and the other *A. viridinutans* strains (cluster VII in Fig. 2). Similarly, many Australian black *Aspergillus* isolates displayed unique mitochondrial DNA restriction profiles compared with those collected from other parts of the world [24]. These observations have been

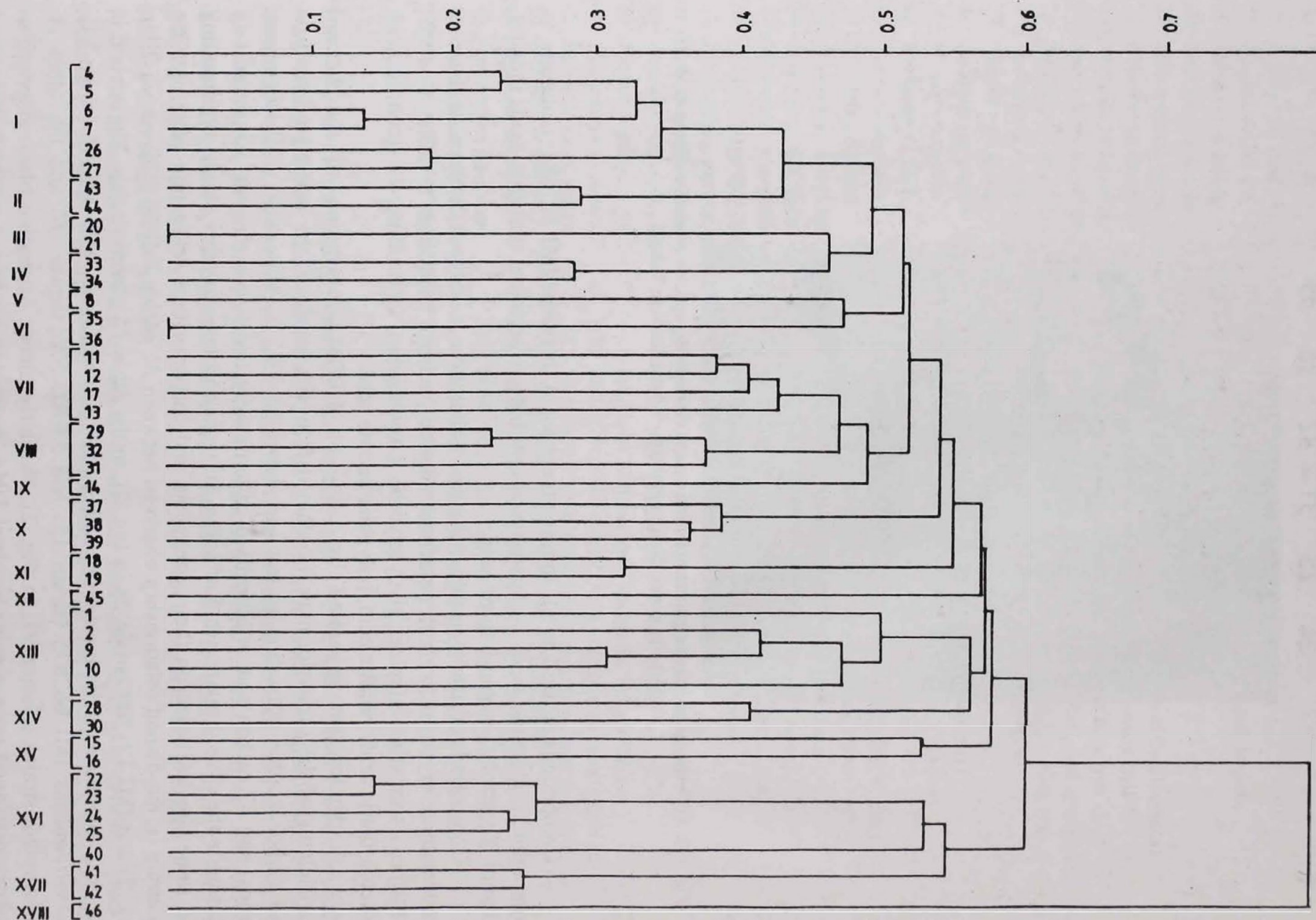


Fig. 2. UPGMA dendrogram based on isoenzyme and carbon source utilization patterns of strains representing *Neosartorya* species and their asexual relatives. The scale represents dissimilarity (squared distance). Strain numbers are those listed in Table I.

The clusters are detailed in the text

unexpected. It is tempting to conclude that Australian strains evolved at a different rate than those from other locations because of the geographical isolation of the continent. Many other Australian strains should be examined by classical and molecular taxonomic methods to find out unequivocally the reason for the observed peculiarities.

Altogether, 18 clusters could be identified on the dendrogram (Fig. 2). Cluster I includes *A. fumigatus* and *N. fischeri* strains. The 4 *A. fumigatus* strains examined (strains 4–7 in Table I and Fig. 2) could only be distinguished by their growth on melezitose and lysine, and by their β -arylesterase and acid phosphatase patterns as described earlier [14]. The strains examined include the type strains of two "subspecies" of *A. fumigatus*, *A. fumigatus* var. *ellipticus* (NRRL 5109) and *A. fumigatus* var. *acolumnaris* (NRRL 5587). These strains exhibited almost identical substrate utilization patterns, this observation underlines our earlier findings based on isoenzyme analysis, mitochondrial and nuclear DNA restriction patterns and amplified DNA profiles of these strains, that these subspecies are only morphological variants of the *A. fumigatus* species [14]. *A. fumigatus* and *N. fischeri* were described as closely related by Raper and Fennell [2], and by Peterson [5], based on their morphological features, and on their DNA reassociation kinetics, respectively. We found that the *HaeIII*-digested mitochondrial DNA patterns and the *SmaI* digested nuclear DNA patterns probed with the ribosomal repeat unit of *A. nidulans* were non-polymorphic for these two species (J. Varga, unpublished results). Girardin et al. [25] could only distinguish *A. fumigatus* from *N. fischeri* by hybridization with an *A. fumigatus* gene coding for a 33 kD protease, and by sequence analysis of moderately repetitive nonribosomal sequences. We found that *A. fumigatus* strains were unable to utilize lactose and cis-aconitic acid, while the two *N. fischeri* strains tested were able to use these compounds as sole carbon sources. Isoenzyme patterns of these species were also similar (data not shown).

Clusters II, III and IV correspond to the species *N. spinosa* (strains 43 and 44), *N. aureola* (strains 20 and 21) and *N. hiratsukae* (strains 33 and 34 in Table I and Fig. 2), respectively. Both the isoenzyme patterns and the substrate utilization spectra of these species were distinctive.

Cluster V involves "*A. fumigatus*" FRR 1266 (strain 8), while cluster VI consisted of the two *N. pseudofischeri* strains (strains 35 and 36 in Table I and Fig. 2). Morphological examination of the former strain led to the conclusion that it is an unusual *A. fumigatus* isolate, although its isoenzyme patterns, mitochondrial DNA and ribosomal DNA profiles, as well as random amplified polymorphic DNA patterns were unique among the *A. fumigatus* strains examined [14]. This isolate could also be distinguished from other *A. fumigatus* strains by its unique ability to assimilate galactitol, lactose, adonitol, melezitose or cis-aconitic acid as sole carbon source. This strain differed from the *N. pseudofischeri* strains both in its inability to use citrulline or vanillic acid as sole carbon source, and in its isoenzyme patterns. The observation, that the *N. pseudofischeri* strains are the most closely related ones to strain FRR 1266 is not unexpected, since their isoenzyme and amplified DNA profiles were also found to be similar [14]. The two *N. pseudofischeri* strains revealed identical substrate utilization and isoenzyme patterns.

Cluster VII includes 4 *A. viridinutans* strains (strains 11, 12, 13 and 17 in Fig. 2). The evolutionary distance among these isolates was much higher than those observed in most of the other species examined.

Cluster VIII involves three *N. glabra* strains (strains 29, 31 and 32 in Table I and Fig. 2). Strains NRRL 2163 and IMI 061447 represent the same isolate maintained in two different culture collections, and could only be differentiated by their slightly variable β -arylesterase profiles (data not shown).

Cluster IX corresponds to one Australian *A. viridinutans* strain (IMI 306135), while cluster X includes the *N. quadricincta* strains (strains 37, 38 and 39 in Fig. 2).

Cluster XI contains *N. aurata* strains (strains 18 and 19 in Fig. 2), while cluster XII corresponds to *N. stramenia* NRRL 4652 (strain 45 in Fig. 2). This strain was found to be closely related to the two *N. aurata* strains as regards their carbon source utilization patterns, but displayed unique isoenzyme profiles. These species were proposed to be closely related based on their color and macromorphology [4], ascospore ornamentations [8], and mitochondrial and ribosomal DNA restriction patterns [26].

Cluster XIII is composed of strains representing three species, *A. brevipes* (strain 1), *A. duricaulis* (strains 2 and 3) and *A. unilateralis* (strains 9 and 10 in Table I and Fig. 2). These strains belong to the same cluster based on both their isoenzyme and substrate utilization patterns (data not shown). The close relationship of these species is in agreement with the proposal of Kozakiewicz [11], who considered these strains as conspecific, based on their similar conidial ornamentations (*A. unilateralis* was reclassified as a subspecies of *A. brevipes*, while *A. duricaulis* was considered to be a synonym of *A. brevipes*). Samson et al. [8] detected species-specific secondary metabolite profiles in the cases of these strains, although the two *A. duricaulis* strains (IMI 217288 and NRRL 4021) displayed different secondary metabolite profiles.

Cluster XIV includes two *N. glabra* strains of Australian origin (strain numbers 28 and 30 in Fig. 2). These two strains are unrelated to the other *N. glabra* strains examined (cluster VIII in Fig. 2). The heterogeneity of the *N. glabra* species was also observed by Samson et al. [8], who established three chemotypes within this species based on their secondary metabolite profiles. Girardin et al. [25] also observed high levels of variability among the *N. glabra* strains examined based on their nuclear DNA restriction patterns.

Cluster XV consists of two Australian *A. viridinutans* strains, NRRL 576 and IMI 062875. These strains represent the type strains for the species (NRRL 4365) [4] maintained in two different culture collections. However, NRRL 576 was found to be morphologically different from *A. viridinutans* IMI 062875, producing abundant conidia. These strains are only distantly related to each other as far as their isoenzyme profiles and substrate utilization patterns are concerned.

Cluster XVI involves the 4 *N. fennelliae* strains examined (strain numbers 22–25 in Table I and Fig. 2) and the "*N. glabra*" strain NRRL 4179 (strain number 40), while cluster XVII is composed of the two *N. spathulata* strains (strain numbers 41 and 42 in Table I and Fig. 2). Strain NRRL 4179 was described by Peterson [5] as a possible representative of a new *Neosartorya* species. Its DNA reassociation kinetics was most similar to those of the *N. fennelliae* strains. The substrate assimilation spectra of these strains are also similar. Furthermore, hybridization with the mating type genes and flanking regions of *Neurospora crassa* resulted in the same patterns in *N. fennelliae* and *Neosartorya* sp. NRRL 4179 (J. Varga, unpublished results). Hybridization was also observed to the DNA of *N. spathulata*, but none to any of the other *Neosartorya* species examined. The hybridizing DNA fragments are possibly homologous to the flanking

regions of the mating type genes, since mating type idiomorphs of *Neurospora crassa* were not homologous to *N. fennelliae* DNA [27]. In view of the distant relationship of *N. fennelliae* to *A. fumigatus* based on their DNA reassociation kinetics [5], isoenzyme profiles and carbon source utilization patterns (Fig. 2), it is quite unexpected that *N. fennelliae* NHL 2951 (mating type A) produced abortive cleistothecia when paired with some *A. fumigatus* strains [28].

Cluster XVIII contains *A. cervinus* IMI 107684 (strain number 46 in Table I and Fig. 2), the strain used as outgroup in these experiments. This species was earlier found to be unrelated to section *Fumigati* based on the composition of its ubiquinones, which consisted of 9 isoprene units, in contrast to the ubiquinones containing 10 isoprene units characteristic to all species of section *Fumigati* [29]. We also planned to use an *A. clavatus* strain (SZMC 0918) as outgroup, which belongs to section *Clavati*, and has ubiquinones consisted of 10 isoprene units [29]. Based on their carbon source utilization patterns, *A. clavatus* was found to be more closely related e.g. to *A. fumigatus*, than *N. aurata*, or the heterothallic *Neosartorya* strains (data not shown). Samson [4] proposed that sections *Clavati* and *Fumigati* are closely related, based on the observation that they produce some common secondary metabolites. However, the *A. clavatus* strain examined did not display any common isoenzyme bands with strains of section *Fumigati*.

As a summary, isoenzyme analysis, and carbon source utilization patterns were found to be valuable tools for the taxonomy of section *Fumigati* in the genus *Aspergillus*. Most species formed well-defined clusters based on their isoenzyme and carbon source utilization patterns, with the exceptions of *N. glabra* and *A. viridinutans* strains; isolates representing these species were highly polymorphic. Australian isolates of these species were found to be only distantly related to strains isolated from other geographical locations. Asexual and sexual species are scattered through the dendrogram. *A. fumigatus*, "*A. fumigatus*" FRR 1266 and four *A. viridinutans* strains were found to be the most closely related to *N. fischeri*, *N. pseudofischeri* and *N. glabra* strains, respectively (Fig. 2), indicating that loss of meiosis might have taken place several times during the evolution of this section. Similar conclusions were drawn by LoBuglio et al. [30], who examined sexual *Talaromyces* and asexual *Penicillium* species.

Further studies are in progress to examine the applicability of carbon source utilization and isoenzyme patterns for the taxonomy of other sections of the *Aspergillus* genus (sections *Circumdati* and *Nidulantes*), and to compare the results with those obtained by using genotypic (mitochondrial and nuclear DNA restriction patterns, and amplified DNA profiles) approaches.

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Abbreviations: ATCC, American Type Culture Collection, Rockville, Maryland, USA; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; FRR, CSIRO Food Research Culture Collection, North Ryde, New South Wales, Australia; IM, International Mycological Institute, Egham, Surrey, UK; NHL, National Institute of Hygienic Sciences, Tokyo, Japan; NRRL, Agricultural Research Service Culture Collection, Peoria, Illinois, USA.

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STUDIES ON THE COMPOSITION AND PROPERTIES OF THERMOPHILIC *BACILLUS* POPULATIONS IN SUBTERRANEAN THERMAL WATERS OF BUDAPEST (HUNGARY)

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A collection of 148 thermophilic *Bacillus* isolates were obtained from two thermal wells (water temperature at about 70 °C) in the area of Budapest (Hungary). These isolates, furthermore seven authentic thermophilic reference strains of *Bacillus* spp. were submitted to detailed comparative biochemical-physiological studies. Overall similarities of these strains for 134 unit characters were determined by the S_{SM} coefficient and clustering achieved using the UPGMA algorithm. According to the results of our computer aided numerical taxonomic analyses, numerous metabolic types, varieties and mutants of a single *Bacillus* sp. not identical with the known thermophilic *Bacillus* species constitute the major fraction of the indigenous thermophilic microbial communities in the thermal waters of the Pascal and Széchenyi wells. The data also revealed, (1) that the most adapted varieties of this *Bacillus* sp. can form the most dense local populations in these subterranean ecological systems. Furthermore, it seems to be probable (2) that the cells of the most commonly occurring varieties of this *Bacillus* sp. may be able to migrate in the underground thermal water systems between diverse subterranean regions located more or less far from one another.

The number of studies on microorganisms from extreme environments, especially on bacteria from thermal environments has considerably increased during the recent years. Although the list of thermophilic genera and species of procaryotes is permanently broadening, the possibilities of a correct intrageneric differentiation among the thermophilic members of the genus *Bacillus* can encounter, mostly, in insuperable difficulties.

It is more than 100 years since Miquel [1] isolated a thermophilic aerobic endospore-forming bacteria from the river Seine. This organism was able to grow at 73 °C and was named *Bacillus thermophilus*. The sixth edition of Bergey's Manual of Determinative Bacteriology [2] characterised 18 species of thermophilic *Bacillus* able to grow at or above 60 °C and listed further 30 ones which were specifically not characterised. Based on the work of Gordon and Smith [3] in the seventh edition of

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Bergey's Manual [4] has been considerably simplified the specific level differentiation listing only *B. stearothermophilus* and *B. coagulans* as true thermophilic species of *Bacillus*. In Bergey's Manual of Systematic Bacteriology [5] seven species (which are able to grow at 55 °C) were accepted: *B. acidocaldarius*, *B. brevis*, *B. coagulans*, *B. licheniformis*, *B. schlegelii*, *B. stearothermophilus* and *B. thermoglucosidasius*. Further 7 species were included in the list of "Species Incertae Sedis". The taxonomy of thermophilic bacilli has been reviewed during the last ten years by Logan and Berkeley, Berkeley et al., Stackebrandt et al., Sharp et al., Priest et al., Priest and Alexander, De Bartolomeo et al., Ash et al., Rössler et al., Berkeley and Goodacre, White et al. and Farrow et al. [6–17]. The increasing number of proposed, suggested or described species in the published works of these authors corroborate the fact that the very large population of thermophilic bacilli in the nature may be much more differentiated than originally considered. This is the reason that according to the opinion of the majority of *Bacillus* taxonomists (e.g. F. G. Priest, R. J. Sharp, D. White), we are able at present to identify generally at species level only a small fraction of the freshly isolated thermophilic bacilli.

The aim of our below presented studies was to clarify the occurrence and distribution of thermophilic bacteria in the subterranean thermal waters in the area of Budapest and to characterise the most frequently occurring thermophilic microorganisms isolated from three thermal wells (Széchenyi II., Magda and Pascal) of about 70 °C temperature.

Materials and methods

The hot subsurface karst waters, dominated by hydrogencarbonates and sulphates, of the studied thermal wells derive directly or indirectly from karstic Triassic formations. These four wells (from among the more than one hundred springuprushers in Budapest) are not only belonging to the very same water quality-category based on their chemical characteristics but a hydrological experiment made in 1966 [18, 19] could prove their underground connections, too. During this experiment in one of the four wells the water exploitation was strongly increased in order to achieve an artificial water pressure level depression. The pumping was continued for 15 days and depression rates were measured in eight adjacent wells supposed to have connection. In case of only three wells became evident the depression immediately or some hours later. In the other five wells there was no significant change in the water pressure levels. Accordingly, the investigated four wells seem to be interconnected via the water-bearing Triassic strata.

In case of each well, water is rising to the surface with a considerable pressure. The steel wellheads are supplied by sampler valves and at sampling we followed the traditional aseptic water-collecting method: before collecting of the water the surface of all metallic constituents of the tape were sterilized by flame and the water was let to flow away during at least five minutes. Water samples collected in Erlenmeyer flasks have been elaborated still at the sampling day.

Three different methods were employed for the isolation of thermophilic bacteria: direct plating, membrane-filtration technique and enrichment method. All of these methods have been associated with the use of the same five isolation-media composed of different nitrogen and organic carbon sources with low concentration: a) from the well-water-prepared simple "water-agar", b) yeast extract starch medium, c) meat-extract peptone medium, d) MD-medium [20], e) J-medium [21]. The direct plating and membrane filtration techniques proved to be unsuccessful. No colonies or only single

colonies were obtained on the surface of different agar plates. Only the enrichment method was applicable: equal amounts of water samples were aseptically mixed with sterile nutrient solutions. Subsequent to one week incubation at 70 °C samples from enrichment cultures were serially diluted and plated on adequate agar media. The developed colonies were isolated in a random manner.

The differential-diagnostic properties studied were: Gram staining; endospore-staining; catalase production; anaerobic growth; oxidase activity; oxidative and fermentative decomposition of glucose and lactose; nitrate reduction; growth at pH 5.0, 5.7, 6.8 and 10.0; growth with lysozyme present; growth in Difco Azid Dextrose Broth; hydrolysis of casein, gelatine and starch; Voges-Proskauer test and pH in V-P broth; degradation of tyrosine, xantine and hippurate; H₂S production from peptone; sulphatase, phosphatase, DNase and RNase activities; litmus-milk test; growth on MacConkey agar; growth in NaCl 2%, 5%, 7% and 10%; methylene-blue reduction in tryptone broth; hydrolysis of Tweens (-20, -40, -60 and 80); degradation of cellulose; growth on Simmons citrate agar; hydrolysis of esculin; acid production from carbohydrates (L-arabinose, dextrin, ethanol, fructose, D-glucose, glycerol, inositol, D-mannitol, mannose, methanol, ribose, sorbitol, sucrose and D-xylose); gas from glucose; utilization of carbon compounds (L-arabinose, dextrin, dulcitol, ethanol, fructose, galactose, glycerol, glucose, inositol, lactose, D-mannitol, mannose, methanol, raffinose, rhamnose, ribose, sorbitol, sucrose, trehalose, D-xylose, Na-acetate, diNa-benzoate, triNa-citrate, Ca-lactate, diNa-malonate, Na-oxalate, Na-propionate, Na-pyruvate and diNa-tartrate) as sole source of carbon in synthetic basal medium; production of indole; deamination of phenylalanine; formation of dihydroxyacetone; egg-yolk lecithinase reaction; utilization of amino acids (DL-alanine, L-arginine, L-histidine, L-lysine, DL-valine) as sole sources of carbon and nitrogen on synthetic basal medium; arginine dihydrolase test; β -galactosidase (ONPG) test; resistance against antibiotics and antibacterial substances (ampicillin 20 μ g, bacitracin 1 IU, chloramphenicol 30 μ g, colistin 20 μ g, erythromycin 10 μ g, gentamicin 20 μ g, kanamycin 30 μ g, lincomycin 10 μ g, novobiocin 5 μ g, oleandomycin 30 μ g, oxytetracycline 30 μ g, penicillin 3 IU, polymyxin-B 15 μ g, spiramycin 30 μ g, cotrimoxazole 25 μ g, streptomycin 30 μ g, tetracycline 30 μ g, vancomycin 50 μ g).

Detailed descriptions of the above listed methods are published in the works Claus and Berkeley [5], Cowan and Steel [22], Gordon et al. [21] and Szabó [23]. The incubation temperature was 60 °C in all tests.

Results

From the point of view of the growth and activities of microbes in thermal waters the temperature and the O₂ content are key parameters. The water temperature of the wells were between 68 and 72 °C, while the presence of oxygen dissolved in the water was not detectable at all by using the method of Winkler.

One hundred strains isolated from enrichment cultures of the water of Magda well died out after isolation except one which was kept for taxonomic studies. One hundred and fortyeight isolates were obtained from the waters of well Széchenyi II, and Pascal altogether. These all isolates (149 ones) were checked for purity and as representative strains were subjected to detailed differential-diagnostic analyses. All of these strains proved to be extremely similar according to their macro- and micromorphological characters. Their regular rods were 0.8 to 1.0 μ m in diameter and the length of them varied between 3.0 to 5.0 μ m. Their endospores (0.8–1.0 μ m $\times$ 1.0–2.5 μ m) were located terminally or subterminally. Since all strains proved to be facultatively anaerobic Gram-positive sporeforming rods, they belong to the genus *Bacillus* according to the latest

edition of Bergey's Manual of Systematic Bacteriology [5]. For simultaneous comparative studies with our isolates we obtained 7 authentic thermophilic *Bacillus* strains from the German Collection of Microorganisms (DSM). All strains (our isolates and the authentic ones) were characterized by using the very same set of differential diagnostic properties. The results were coded into 134 characters for computerized numerical analyses. Similarity calculations were based on the simple matching coefficient of Sokal and Michener and clusters were formed by the unweighted pair group method with arithmetic averages (UPGMA), (Fig. 1).

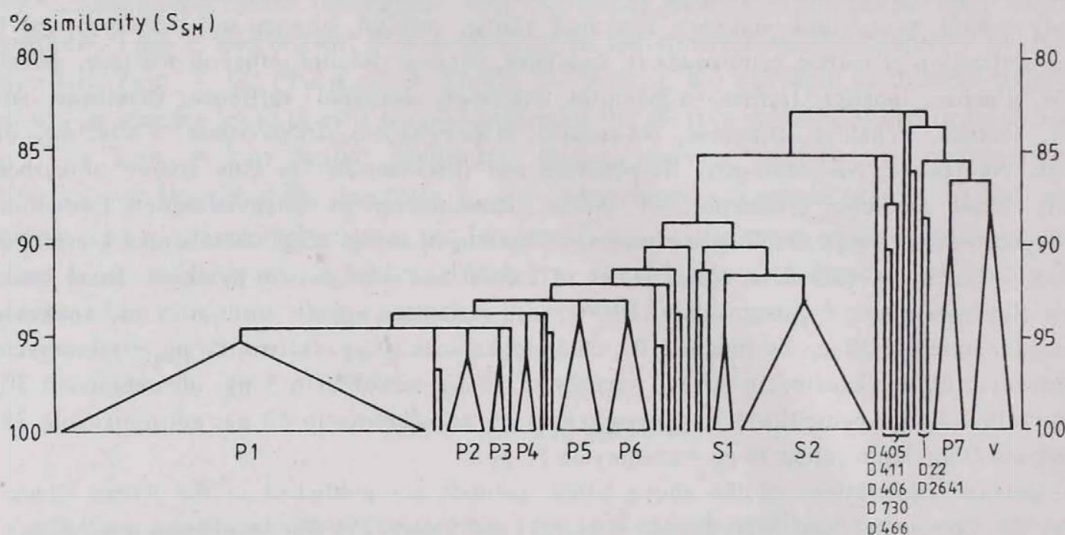


Fig. 1. Simplified dendrogram showing percentage similarity of 149 thermal water strains and 7 authentic thermophilic *Bacillus* strains involved in simultaneous comparative studies based on 134 coded characters. (D405: *B. caldolyticus*, D411: *B. caldovelox*, D406: *B. caldotenax*; D730: *B. thermocatenulatus*, D466: *B. thermodenitrificans*, D22: *B. stearothermophilus*, D2641: *B. flavothermus*, P1–P7: these phenons are formed of strains originating from Pascal well; S1 and S2: comprising the strains isolated from Széchenyi II well; V: comprising strains isolated from Pascal, Széchenyi II and Magda wells

In the presented simplified dendrogram it can be seen that our *Bacillus* strains have a considerable phenetic heterogeneity. One hundred and thirty-five strains out of the total 149 *Bacillus* cultures were united (at the left of the dendrogram) at 85.5% average similarity level separating the seven authentic thermophilic *Bacillus* strains and two small phenons of our isolates into a larger mixed group of strains (at the right of the dendrogram). The big group with 135 strains comprise 8 phenons and 15 solitary strains in intergroup positions. P1 and S2 out of these phenons represent dominant clusters each of them composed only by Pascal well and Széchenyi II well strains, respectively. (From P1 to P7 phenons are formed of strains originating exclusively from Pascal well. Phenons denominated as S1 and S2 comprise Széchenyi II well-water strains. Seven strains were

clustered together in a heterogeneous mixed group, denominated as V, which comprise Széchenyi II and Pascal well strains together with the only one strain isolated from well Magda.)

Table I

Percentage of positive results (and the number of positive strains) regarding 16 selected differential-diagnostic properties in the individual separated phenons (see Fig. 1)

	P1	P2	P3	P4	P5	P6	S1	S2	P7	V
Number of strains $\Sigma 133$	63	7	3	4	8	8	4	23	6	7
Hydrolysis of starch	10 [6]	0	66 [2]	0	63 [5]	0	100 [4]	96 [22]	83 [5]	86 [6]
Degradation of tyrosine	98 [62]	57 [4]	100 [3]	25 [1]	100 [8]	100 [8]	0	17 [4]	16 [1]	14 [1]
Hydrolysis of casein	19 [12]	0	0	0	0	0	100 [4]	70 [16]	0	43 [3]
Hydrolysis of esculin	62 [39]	71 [5]	66 [2]	100 [4]	25 [2]	88 [7]	100 [4]	100 [23]	16 [1]	14 [1]
Hydrolysis of Tween 80	5 [3]	29 [2]	0	50 [2]	0	13 [1]	75 [3]	74 [17]	67 [4]	71 [5]
Production of H ₂ S	37 [23]	14 [1]	100 [3]	0	38 [3]	50 [4]	0	17 [4]	33 [2]	0
Oxidative utilization of lactose	37 [23]	29 [2]	66 [2]	0	75 [6]	63 [5]	0	0	0	71 [5]
Acid from mannitol	5 [3]	0	66 [2]	25 [1]	50 [4]	0	0	100 [23]	100 [6]	100 [7]
Acid from arabinose	100 [63]	100 [7]	100 [3]	100 [4]	100 [8]	100 [8]	100 [4]	91 [21]	0	0
Acid from glycerol	10 [6]	0	0	0	0	0	75 [3]	87 [20]	0	28 [2]
Acid from xylose	98 [62]	86 [6]	100 [3]	100 [4]	100 [8]	88 [7]	75 [3]	100 [23]	0	0
Utilization of ethanol	100 [63]	100 [7]	100 [3]	100 [4]	100 [8]	100 [8]	0	100 [23]	16 [1]	0
Utilization of lactose	98 [62]	100 [7]	100 [3]	100 [4]	100 [8]	100 [8]	100 [4]	61 [14]	0	0
Utilization rhamnose	97 [61]	100 [7]	100 [3]	100 [4]	100 [8]	100 [8]	0	0	16 [1]	0
Utilization of arginine	5 [3]	100 [7]	0	100 [4]	0	0	100 [4]	100 [23]	83 [5]	14 [1]
Gas from NO ₃	98 [62]	86 [6]	100 [3]	75 [3]	100 [8]	100 [8]	0	0	0	0

Investigating the taxonomic position of these separated phenons in details, in respect of all strains, 30% of the tests proved to be equally negative: sulphatase and urease activity; V-P test; litmus-milk test; growth on MacConkey agar; methylene-blue reduction; growth on Simmons citrate agar; production of indole; degradation of cellulose; growth in NaCl 7% and 10%; deamination of phenylalanine; formation of dihydroxyacetone; degradation of hippurate; egg-yolk lecithinase reaction; gas from glucose; arginine dihydrolase test; utilization of benzoate, malonate, oxalate, propionate and tartrate as sole sources of carbon; utilization of DL-alanine and DL-valine as sole sources of carbon and nitrogen. This by no means seems to be methodological fault, since most of these test methods were used successfully in other experiments. Only the results of approximately sixteen tests can be considered as having differential value which are depicted in Table I.

Tyrosine was degraded only by strains of P groups whereas S1 and S2 strains did not hydrolyse this compound. On the contrary, hydrolysis of casein was negative only with P strains. The P1–P6 phenons were distinguished from S phenons by acid production from glycerol, utilization of rhamnose, gas formation from nitrate, hydrolysis of starch and Tween 80. Acid production from arabinose and xylose, utilization of ethanol and lactose were approximately 100% positive by all strains of left-side group of dendrogram contrasted with P7 and V phenons. In case of the other tests the deviation of phenons' properties was independent of originating of strains.

Discussion

The detected aspects of the studied diagnostic characteristics of the *Bacillus* strains isolated by us differed considerably from all of the descriptions given by the Bergey's Manual [5] or White et al. [16] for the presently known thermophilic *Bacillus* species. This fact was corroborated by the results of our computer analysis, too. However on the basis of our data demonstrated in the dendrogram we can assume that the phenons P1–P6 and S1–S2, furthermore the 15 strains located by the computer in intergroup positions among them (on the left of the dendrogram) represent different ecotypes of an unknown *Bacillus* species adapted to the minor differences in the geological and physicochemical parameters of the two wells. P1 and S2 are the most adapted varieties (or already species) of this till now unknown *Bacillus* taxon which can form the most dense local populations in the Pascal and Széchenyi II wells respectively. Furthermore it seems to be probable that bacilli may be able to migrate in the underground thermal water-systems. The strains of S2 phenon include also a strain from Pascal well. Presumably this P strain originates from the thermal water-system of Széchenyi II well considering the proximity of the wells. This underground connection to each other was proved with hydrological investigation as explained earlier. Our isolates placed into the phenons No. P7 and V represent probably two further, less adapted and less common *Bacillus* spp.

Summing up we can conclude that the waters and the water bearing strata of the Pascal and Széchenyi II wells are colonized by very large populations of an up till now less studied variable thermophilic *Bacillus* species which is capable of migrating through these underground water systems and forming locally adapted subpopulations in particular subterranean surroundings.

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NEW DRUG EFFECTS ON BACTERIA, THE ANTIPLASMID EFFECT*

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Science provides methods of control for the problems inherited from past generations, but it cannot prepare solution for specific problems of tomorrow because it cannot predict what the problems will be. Physicians and public health officers, like soldiers, are always equipped to fight the last war as it was written by R. Dubos more than 40 years ago [1]. However things changed and today we know several diseases to fight against like cancer, AIDS or antibiotic resistant infections. In bacterial, viral infections and cancer cases resistance appeared in the last decades, but only a limited number of new drugs are being developed. WHO has estimated several millions of bacterial infections and over 10 million new cases of cancer last year. A large proportion of patients with infections do not respond the drug treatment because of the emerging drug resistance of the pathogenic bacteria.

Professor Manninger who synthetised the previous knowledge was one of the greatest Hungarian microbiologists with numerous findings in bacteriology and immunology. One of his main work, the virulence of *Bacillus anthracis* was the favourite pet of another great microbiologist George Ivánovics, who had an analytic way of thinking, directed my PhD thesis and watched my steps in medical microbiology. My PhD thesis concerned with pathogenicity, virulence factors of *Bacillus anthracis*. When my thesis was about completed we had a serendipity by recognizing the effects of well known medicines on destabilization of extrachromosomal genetic elements, plasmids of bacteria. Theoretically the elimination of antibiotic resistance plasmid was promising, because nearly 50 000 men, women and children were dying every day from infectious diseases, there was no treatment for many of these diseases as a consequence of antibiotic resistance. Even today, WHO report drug resistant strains of microbes having an impact on the fight against tuberculosis, cholera, diarrhea and pneumonia – major diseases which together killed more than ten million people in 1995. Some bacteria are resistant to as many as ten different drugs.

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What is the reason for this type of antibiotic resistance? In most cases the plasmid encoded – so called infectious antibiotic resistance – is responsible for the multidrug resistant bacteria in our environment as a consequence of the uncontrolled and inappropriate use of antibiotics globally (e.g. antibiotics are used in enormous amounts worldwide e.g. some 170 billion tons of animal meat produced every year for human consumption).

The plasmids make bacteria more flexible in their adaptation to the environmental changes, e.g. they are able to grow in the presence of antibiotics.

The development of antibiotic resistance involves a wide variety of plasmid encoded mechanisms: 1. enzymes metabolize antibiotics before they can exert their action, 2. changes in the target molecules, 3. impermeability barriers, and 4. operating efflux pump transporter proteins. These factors reduce the intracellular accumulation of chemotherapeutics, resulting in ineffective treatment.

Bacteria resistant to many unrelated antibiotics often cause serious therapeutic failure when plasmids make the bacteria insensitive or resistant, and the patient become incurable with the usual antibiotics. Nevertheless we are seeking drugs against polyresistant bacteria, i.e. drugs against plasmids as the genetic basis of antibiotic resistance. Possible drugs include the well-known phenothiazines, which have been rediscovered during the last 20 years. The great finding that methylene blue has an antimicrobiological effect was published a hundred years ago by Gutmann and Ehrlich [2], while the antibacterial activity of phenothiazines was demonstrated in the 1930s [3–5]. Laborit described the action of phenothiazines on the central nervous system as pain killers in 1949 [6], and the action of chlorpromazine as a tranquillizer was discovered in 1952 [7].

Later the antibacterial effects of phenothiazine compounds were systematically tested on a wide variety of Gram-positive and Gram-negative bacteria [8–13]. It was demonstrated that the basis of the antibacterial effect was an increased permeability of the cell wall [13,11].

Many tricyclic psychopharmaca with an antibacterial effect eliminate the resistance and metabolic plasmids of *Escherichia coli* and other bacteria. Antibiotic resistance of bacteria can spread in three different levels: 1. antibiotic resistant bacteria transfer from one person to another, 2. plasmids mediate the resistance among bacteria and finally, 3. transposons can transfer the resistance markers from plasmid to bacterial chromosome.

This action on bacterial plasmids is rather specific and depends on the chemical structure of “antiplasmid compounds”. Some drugs have only an antibacterial effect. *E. coli* plasmids are eliminated by several tricyclic psychopharmaca, e.g. phenothiazines, stereoisomers of thioxanthenes and enantiomers of mepromazine [16–20]. Besides the R plasmids of *E. coli*, *Proteus vulgaris*, *Klebsiella pneumoniae*, the virulence plasmids of *Yersinia*, *Agrobacterium tumefaciens* and *E. coli* strains [21], other, e.g. haemolysin plasmids were also eliminated with varying frequencies.

The same plasmids were eliminated with different frequencies from different *E. coli* strains.

The F⁺lac plasmid of *E. coli* served as a convenient model to study the plasmid-curing effects of various tricyclics, because lactose plasmid-carrying colonies can easily be differentiated from plasmid-less pink colonies. In these experiments the plasmid

content of the cured bacteria was determined on EMB agar. It was found that the phenotypically lac-negative bacteria, which had lost the lactose fermentation ability, had, in fact, lost the plasmid DNA as well. The latter was analysed by agarose gel electrophoresis. The disappearance of the plasmid DNA content of the bacteria provided firm evidence of plasmid curing.

One purpose of our study was to clarify the exact mechanism of action of the drugs on plasmid replication. The results revealed that antiplasmid compounds have membrane effects [22]. The ordered replication and segregation of plasmids are essential if they are not to be lost from their host cell line. The minimum requirement for the independent replication of the plasmid from the chromosome, is the origin of replication, where the vegetative replication is initiated. The transforming activity of plasmid DNA was inhibited in the presence of a low concentration (10/ μ g/ml) of tricyclic compounds [23]. The results show that the replication of plasmid DNA, partition and intercellular plasmid transfer – the most sensitive targets for the drugs – could be inhibited simultaneously.

This effect of the psychopharmaca showed a good correlation with membrane effects, as demonstrated by chromium uptake experiments. The ^{51}Cr uptake was increased on increase the promethazine concentration. We believe that there is competition between bivalent cations and antiplasmid compounds in the membrane. Evidence was found on reactor neutron activation analysis (Prof. Henrik Rausch) to confirm the hypothesis that tricyclic psychopharmaca displace a portion of the Ca^{++} and Mg^{++} from the bacterial cells. It was pointed out that one molecule of Ca was displaced by two molecules of CPZ [24, 25].

This process leads to the depolarization on the membrane. The perturbation induced in the membrane may be responsible for biological effects. In the previous experiments it was found that the antiplasmid compounds were able to inhibit the stability of the plasmids in the transformed cells.

One of the key enzymes of plasmid replication is the plasmid DNA gyrase. Several inhibitors of this enzyme were tested as regards the plasmid-curing effect of the tricyclic psychopharmaca. Novobiocin (which acts on the B subunit of DNA gyrase) enhanced the antiplasmid effects of some tricyclic drugs. When the compounds were applied at ineffective concentrations, a synergism was found between Novobiocin and the drugs in the antiplasmid effect.

It was presumed that the drugs form an intracellular, intermediate complex with plasmid DNA and gyrase. As a result of the action of promethazine on plasmid DNA, a complex was formed. Our results show that the supercoiled form is missing, the intensity of the relaxed circular form has decreased and the linear form of plasmid DNA can be found in the best visible band of agarose gel electrophoresis on increasing the concentration of promethazine.

The complex formation between the high concentration of promethazine and CCC (covalently closed circular) form of plasmid DNA and inhibition of intercellular plasmid transfer are probably responsible for the plasmid elimination, which is limited to a small part of population.

Attempts were made to improve the antiplasmid effects of tricyclic drugs by computer-aided drug design because the effective concentrations in vitro were much higher than the achievable serum concentrations in patients. Study of the structure activity

relationship in in vitro experiments demonstrated that changes in the molecular structure of the tricyclic compounds modified the antiplasmid activity of the compounds (S. Földeák). Studies by Szent-Györgyi [26] and Pullman and Pullman [27] indicated that the biological effects of phenothiazines may depend on their ability to form electron donor-acceptor complexes. Calculations of the energies of the highest occupied and lowest unoccupied molecular orbitals of phenothiazines and related compounds show that one molecule has at least two binding sites [28]. Disruption of the pi-electron system of the three rings caused a significant decrease in the antiplasmid effect [29, 30].

In a further study, the antibiotic sensitivity of bacterial strains isolated from patients who regularly received tricyclic psychopharmaca was compared with that of bacteria from patients who did not. There was some differences between the groups of patients in the occurrence of multiresistant bacteria. However, 12 of the 50 drug-treated patients had bacteriuria, whereas 21 of the 50 control patients had significant bacteriuria (Klára Domonkos).

In a large number of experiments a heterogeneity of the bacteria isolated from the drug-treated patients was found. The antibiotic resistance of *E. coli* and *P. vulgaris* was not homogeneous as concerns 10 different colonies isolated from the urine of promethazine- and clozapine-treated patient. The antibiotic resistance of *E. coli* and *P. vulgaris* colonies isolated after a five-day treatment with tricyclic drugs was often changed, but in some cases the resistance was not altered.

When the antiplasmid effect of maprotiline was examined in two patients, it was found that the resistance patterns of five different isolates in the urine from each patient were different to some representative antibiotics.

Some plasmid elimination might occur. Carbenicillin resistance was cured in 1 isolate and cefuroxime resistance was cured in 3 isolates.

The first successful plasmid elimination by promethazine in human patients were described in 1982 [30, 32].

The curing effect of oral ciprofloxacin therapy on the plasmid-bearing strain of *Serratia marcescens* in vivo was reported by Mehtar et al. [20]. In *E. coli* a similar curing has been reported [31, 32]. These studies were followed by the extensive screening of in vitro plasmid eliminating agents [12, 33, 34, 29, 35, 21, 23, 36–39].

To summarize the results, antibacterial actions of some phenothiazines were shown for several bacteria, involving changes in the morphology, biochemical pathways, adhesion properties and homogeneity of the bacterium population. In the bacterial cell, the plasmid replication was selectively inhibited by some tricyclic drugs.

This antiplasmid effect was exerted on the antibiotic resistance, metabolic and virulence plasmids including haemolysin transporter plasmids. We assume that the mechanism of elimination can involve enzyme inhibition in the plasmid replication machinery, where the drugs bind via biological charge transfer complex formation. At any rate, some correlation exists between the electrochemical structure and the antiplasmid effects of the tricyclic compounds [36]. Apart from plasmid curing the synergism between antibiotics and tricyclics could be the consequence of inhibition of ABC transporter proteins like haemolysin transporter when all individual cells of bacterial population were equally sensitive to the efflux inhibitory effect of phenothiazines. Interestingly, a similar synergism between phenothiazines and anticancer drugs in tumour cells was also revealed

[40], therefore some tricyclic compounds can be considered as resistance-reversing agents. We assume that these data may lead to further ideas for the development of more effective combinations in chemotherapy for polyresistant bacterial infections and multidrug resistant cancer.

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**ANNUAL MEETING OF THE
HUNGARIAN SOCIETY FOR MICROBIOLOGY**

NYÍREGYHÁZA, AUGUST 21–23, 1996

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PLENARY SESSION

J. MOLNÁR

Antiplasmid effects of pharmaceutical preparations other than antibiotics

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Some bacterial plasmids undergo spontaneous loss at low frequency. Plasmids can be eliminated by the use of tricyclic drugs to increase the frequency of segregation. The purpose of this study was to clarify the specific mechanism of action of the above antiplasmid drugs on various plasmids. The antibiotic resistance coding plasmids were eliminated from *Escherichia coli*, *Proteus vulgaris*, *Proteus mirabilis*, *Acinetobacter anitratus*, *Klebsiella pneumoniae*, *Agrobacter tumefaciens* and *Staphylococcus aureus* strains with a frequency of 0.2 to 15% by promethazine and amitriptyline. The colicin V production of *E. coli* strains, the pigment production of *Serratia marcescens* and *S. aureus*, the haemolytic activity of some *S. aureus* and *E. coli* cells, the nod plasmid of *Rhizobium meliloti* and the virulence plasmids of *A. tumefaciens* and *Yersinia enterocolitica* were eliminated in the presence of promethazine.

The antiplasmid compounds affect the topological state of plasmid DNA. The superhelical form DNA is diminished and the linear form increases in the presence of antiplasmid compounds. The biological activity of plasmid DNA in transformation is also decreased. The binding affinity of antiplasmid H3 imipramine is ten times higher for the supercoiled form of plasmid DNA than for the linear form. Transconjugal plasmid

transfer is inhibited as well. The process in this way results in plasmid-free bacteria in the population. Quantitative structure-activity relationship studies (QSAR) revealed sole correlations between the antiplasmid effect the symmetry of the HOMO orbitals and the electrophilic superdelocalizability on atoms C8, C9 and N10 of the tricyclic skeleton. Antiplasmid compounds form charge transfer complexes with plasmid replication machinery. The elimination of plasmids conferring pathogenicity and resistance to antibiotics can open up new horizons in experimental chemotherapy.

VIROLOGY

GY. SZŰCS and M. ÚJ

First detection of human astroviruses in Hungary

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Gastroenteritis caused by star-shaped astroviruses in infants was first recognized in 1975. In the last decade, significant advances have been made in adapting the human astroviruses to growth in cell culture, in developing monoclonal reagents to detect these viruses in enzyme immunoassay, and introducing molecular techniques to reveal the viral genom in clinical specimens. These have allowed the identification of astroviruses in outbreaks of gastroenteritis in children and adults worldwide.

The lack of information about the role of astroviruses in gastrointestinal cases in Hungary, we aimed to use a modified ELISA in detecting astrovirus antigens directly in stool samples. (Test reagents and protocol were provided by Dr. John E. Herrmann, Division of Infectious Diseases, Massachusetts University Medical School, Worcester, MA, USA). Five hundred stool samples collected from children with gastroenteritis below 5 years of age were investigated in astrovirus antigen ELISA. Sixty-six (13.2%) of the 500 samples were astrovirus-positive. Six of 9 astrovirus-positive samples were successfully cultured and passed in CaCo₂ cells in the presence of trypsin. Isolates were identified as astroviruses by immunofluorescence and in RT-PCR. Our screening for astroviruses by ELISA and culture in a collection of stool specimens extends our understanding of the role of astroviruses in pediatric diarrhoea in Hungary.

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Identification and grouping of Newcastle disease virus strains by restriction site analysis of a region from the F gene

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Restriction site analysis of more than 300 Newcastle disease virus (NDV) strains was carried out using reverse transcription (RT) followed by polymerase chain reaction (PCR) amplification of the fusion (F) gene. A 75% region of the F-gene was digested with three restriction enzymes generating thirty restriction sites. Physical maps were constructed for all strains and a large genetic variety of NDV strains were revealed. Strains could be classified into six major genetic groups. Restriction site analysis of the matrix (M) gene has confirmed the separation of these groups. Genetic groups of NDV strains reflected a geographical origin or epizootiological relationship or ecology of the host species. Restriction site mapping allowed the identification of vaccine strains, too and to study the epizootiological relationships of natural virulent isolates.

E. WEHMANN, J. HERCZEG, and B. LOMNICZI

Identification of Newcastle disease virus vaccine strains by restriction cleavage site analysis

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Since the 1940s several avirulent (lentogenic) and moderately virulent (mesogenic) vaccine strains of Newcastle disease virus (NDV) have been introduced for the protection against ND. The restriction cleavage site analysis of the reverse transcribed and PCR amplified genomic RNA allowed the identification of NDV vaccine strains. Description of generic markers of commercial vaccine strains made it possible to identify natural field isolates of reduced virulence and to diminish animal experiments necessary for biological characterization of vaccines.

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Characterization of Newcastle disease endemics

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Newcastle disease occurs in endemic forms in several regions (e.g. Africa and the Far East) while in countries, where strict epidemiological control is exercised, the disease is eradicated in a short time if it is introduced mainly during an epizootic. NDV strains derived from endemic regions are considered the sources of epizootics in developed countries. In this work we have characterized a number of NDV strains isolated in endemic regions, using restriction site analysis of the fusion (F) gene. Generic diversity of virus strains were found with individual genetic variety that is characteristic of a particular region. However endemic nature of infection was revealed in regions where new introductions of ND were considered the primary source of infection.

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DNA sequencing and phylogenetic analysis of the protease gene of ovine adenovirus type 3

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At present, there are six ovine adenovirus (OAV) serotypes officially accepted, but no DNA sequence from any of them was ever published. We have decided to partially clone and sequence the DNA of one serotype in order to make phylogenetic comparisons. The genome of OAV-3 was cleaved with restriction enzymes *HindIII* and *PstI*, and the resulting fragments were randomly cloned into plasmid pUC 19. Both ends of each clones were sequenced, and two *HindIII* clones containing the entire gene of the viral protease in opposite insertions were selected for complete sequencing. Additional restriction enzyme cleavage sites identified in the obtained sequences were used to make inner deletions, for further sequencing. In the middle of the cloned *HindIII* fragment, a *PstI* site was recognized, and later the two *PstI* clones overlapping this region were also found, and sequenced from the ends. Finally, without any complicated method, solely with the use of simple steps of random cloning and cutbacks, the DNA sequence of the complete protease gene and the two neighbouring genes (of the hexon and the DNA binding protein) was obtained. The Phylip program package was used for phylogenetic analysis (homology and parsimony), and OAV-3 was demonstrated to be related to subgroup 1 BAVs, and more specifically (i.e. most closely) to BAV-2. We should also mention, that so far BAV-2 is the only subgroup 1 BAV serotype, which had repeatedly been isolated from both, cattle and sheep.

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DNA technology in veterinary diagnostic virology

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Nested polymerase chain reaction (PCR) assays were developed for the diagnosis of the following viruses: porcine parvo, bovine adeno subgr. I and II, suid herpes type 1 (pseudorabies), equine herpes types 1, 2 and 4, bovine herpes types 1 and 4, bovine viral diarrhoea, border disease, hog cholera, Newcastle disease, Sendai, bovine respiratory syncytial, bovine leukaemia, feline coronaviruses, equine arteritis and rabbit caliciviruses. In addition, a general pestivirus detection PCR assay has been developed, which is able to amplify all pestiviruses. The presence of viral genomes was demonstrated in a high variety of clinical specimens by using simple target preparation methods.

To prevent false positive results, due to carryovers and cross-contaminations, specific instruments (tube holders and openers) and laboratory routines were applied. In order to avoid false negative results, internal controls, termed mimics, were constructed and co-amplified with the real targets in the same reaction tubes.

The sensitivity and specificity of the PCR assays and of conventional diagnostic methods were compared by analysis of thousands of samples during the last nine years. The experience verifies that, by applying safe laboratory routines, the nested PCR assays present highly reliable and sensitive novel alternatives for direct detection and identification of viruses.

In addition, restriction endonuclease analysis (REA) and/or nucleotide sequence determination of the PCR products provided valuable means of identification and characterization of the amplified viral genomes. The comparative genome analysis, performed by REA and by nucleotide sequencing, provided novel means to study the genetic relatedness of viruses, to construct phylogenetic trees and to perform studies of "molecular epizootiology". By this way, phylogenetic trees have been obtained for pestivirus, adenovirus, Newcastle disease virus and rabbit caliciviruses.

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Mapping of naturally arising deletions in bovine adenovirus isolates

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There exist five isolates of bovine adenovirus serotype 10 (BAV-10). The prototype strain was isolated in New Zealand, while four additional strains were described in Northern Ireland. The genome size of each isolate seemed to be different. Physical mapping with several restriction enzymes has proven that the differences in the genome

size are caused by differences between map units 76 and 94. The region was molecularly cloned, then subcloned. We have constructed more defined maps of the four Northern Ireland isolates. We have demonstrated that compared to the strain of largest genome, the three isolates contain deletions between map units 85 and 94, and appr. 1000 base pairs (bp) in size. Two out of these three isolates do even contain a second deletion between map units 76 and 85. The size of this second deletion is estimated to be around 1300–1400 bp. For DNA sequencing, the corresponding clones were treated by Exonuclease III, and nested deletions were created. On the basis of the obtained DNA sequence data, the location of the natural deletions was found to be in the genes of the 12.5K and 34K proteins of the E3 and E4 regions, respectively.

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Unusual genome organisation of bovine adenovirus type 4

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Bovine adenoviruses (BAV) were divided into two subgroups on the bases of their different biological and antigenic properties. The accumulating DNA sequence data of BAV-4, the prototype strain of the so-called subgroup 2 BAVs justifies the subgrouping, moreover it also suggests unusual genome organization compared to other mastadenoviruses. Earlier obtained clones of BAV-4 were subcloned according to the detailed physical maps. The two ends of each clones, and some clones entirely have been sequenced. Approximately 15 kb from the 30 kb large genome is sequenced by now. The genes were identified by the homology of the predicted amino acid sequence of the coded protein by computer analysis, and their location on the genome has been determined. The results are presented on physical and genomic maps. Compared to other mastadenoviruses, relatively few differences were found in the structural proteins. Genes of the early regions, however, are sometimes not recognized even by computer analysis. Our most interesting finding so far is the unusual location of the E3 homologue region to the right from the E4 region, instead of the usual place between the genes of pVIII and the fiber.

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Primary induction of human cytotoxic lymphocytes against a synthetic peptide of the HIV-1 protease

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To identify candidate CTL epitopes in the protease of the human immunodeficiency virus type 1 (HIV-1) MN strain, we synthesized 9mer and 10mer peptides containing the HLA-A*0201 binding motif. The binding affinity of the peptides was measured by HLA-A*0201 upregulation on the antigen processing deficient T2 cells. Exogenously added binding peptides stabilize the "empty" MHC molecules and thereby increase the density of HLA molecules on the cell surface, which can be quantified in a flow-cytometer. Peptides with high binding affinity were tested for ability to stimulate primary cytotoxic T-lymphocytes (CTL) from healthy, HIV-negative blood donors. Peptide-specific CTLs were obtained by stimulation with a 9mer (LVGPTPVNI) and a 10mer (VLVGPTPVNI) peptide derived from a highly conserved amino acid stretch in the C-terminal region of the protease. The epitope represented by the two immunogenic peptides was refined with a set of alanine replacement analogues.

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Detection of human herpesvirus 8 DNA in AIDS-associated and other forms of Kaposi's sarcomas

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The presence and prevalence of Kaposi's sarcoma (KS) associated human herpesvirus (HHV)-8 DNA was determined in 33 cutaneous and visceral KS lesions of 20 individuals with various forms of Kaposi's sarcoma. Study included all AIDS-associated KS cases occurring from 1987 to 1995. DNA from 24 cutaneous and visceral KS specimens of 13 AIDS patients, 5 KS specimen of 3 cases of immunosuppressed patients of renal transplantation as well as cutaneous biopsies from 4 classical KS cases was purified with the phenol-chloroform method. KS1 and KS2 primers were used to amplify a 233-bp DNA fragment located from 987 bp to 1219 bp of KS330 Barn, an HHV-8 like DNA sequence by PCR. The PCR products were subjected to Southern blot hybridization with specific radiolabelled probe DNA. Nucleotide changes compared to the original HHV-8 sequence was determined by direct sequencing of the cloned probe DNA.

HHV-8 DNA was detected in 93% of patients with AIDS-associated KS. Positivity of cutaneous and visceral KS lesions, however varied. HHV-8 like DNA sequences have also been detected in various cutaneous and visceral KS lesions of the 3 post-transplant

patients. One skin lesion, however was negative, while liver KS lesion was positive in one patient. All the skin biopsies from the classical KS cases were positive for HHV-8 DNA. As AIDS associated KS occurs in less than 10% of AIDS patients in Hungary, and Kaposi's sarcoma is more than 20% of all post-transplant tumours it may be assumed that an HHV-8 like herpesvirus is circulating in the general population as a persistent infection controlled by the immune system.

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**Epstein-Barr virus infects human term syncytiotrophoblast
in vitro and induces replication of human T-cell leukemia-
lymphoma virus type I in dually infected cells**

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Human term syncytiotrophoblast (ST) cells cultured in vitro could become actively infected with EBV, expressing immediate-early (Zta), early (EA-D) and late (VCA) proteins of the productive replication cycle and releasing infectious virus. No significant differences were found between type 1 and type 2 EBV strains in their ability to infect ST cells. EBV infection of ST targets was mediated mainly by an unidentified cell surface receptor different from CR2. ST cells exhibited restricted permissiveness for HIV-1, whereas no virus expression was found in ST cell infected with HTLV-I alone. Dual infection of ST cultures with EBV and HIV-1 did not influence the replication of EBV or HIV-1. Coinfection of ST cells with EBV and HTLV-I converted HTLV-I infection from an abortive to a permissive one, whereas no changes were found in the replication of EBV in dually infected cell cultures. Simultaneous presence of both viruses in the same cells was detected by double immunofluorescence. Permissive replication cycle of HTLV-I was induced by the Zta transactivator protein of EBV. Our results suggest that interactions between EBV and HTLV-I in coinfecting ST cells in vivo may contribute to the transplacental transmission of HTLV-I.

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**Seropositivity for adenovirus among European cats
(*Felis cati*)**

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Occurrence of adenoviruses and their potential pathological impact have not been investigated in cats so far. The wide-spread presence of adenoviruses in various species makes it probable that carrier state and infection also exist in cats. Adenoviruses may play an aetiological role in feline syndromes of unknown origin or may have to be included in differential diagnostic schemes.

We began the investigation into the presence of adenoviruses in live and dead cats kept under different conditions. As a first approach, antibody detection by ELISA was used and the results were confirmed by indirect immunofluorescent antibody test and agar-gel immunodiffusion test. Purified hexon was employed as antigen, which has a common antigen determinant in most adenoviruses. Serologically (and clinically) negative SPF experimental cats were immunized with adenovirus hexon, and the serum bled after the third immunization was used as positive control. Field sera/plasmas of 296 Hungarian, 96 Dutch and 102 Scottish cats, and 10 serum samples of Italian experimental cats were tested. Seropositivity was found in the field samples in 11, 20 and 10%, respectively. It is interesting that one of the groups of the 10 experimental cats showed marked elevation in infection extensivity, which suggests the possibility of an artificial infection. These results may reflect the natural infection of the feline population(s), and these findings are similar to those found in humans and other species. Currently an adenovirus isolation attempt from feline samples is in progress.

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**Detection and identification of avian mycoplasmas by
polymerase chain reaction and restriction fragment length
polymorphism assay**

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Polymerase chain reaction (PCR) was used to detect avian mycoplasmas. A primer pair designed for the detection of human and rodent mycoplasmal species was examined concerning the ability of detecting further, namely the most important avian mycoplasma species. We found, that with the exception of *Mycoplasma gallisepticum*, each of the examined avian mycoplasmas could be detected by this primer pair, and distinction could

be made among them by restriction fragment length polymorphism (RFLP) assay using two restriction enzymes (*Bam*HI, and *Rsa*I). For the detection of *M. gallisepticum* by PCR we applied species specific primers. Using samples taken by swabbing or homogenizing different organs, specific result can be achieved within one working day in positive cases, thus the method we applied could improve and facilitate the identification of avian mycoplasmas.

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The complete nucleotide sequence of RNA 2 of the cucumber mosaic virus strain TRK7

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The genome of the broad host range cucumber mosaic cucumovirus (CMV) consists of three positive sense RNA molecules. RNA1 and 2 (3.3 and 3.0 kb respectively) carry the genes responsible for the replication of the virus, while RNA3 (2.2 kb) codes for the movement protein and for the coat protein.

The primary structure of RNA3 of a Hungarian CMV strain (Trk7-CMV) was determined in an earlier work. In this study, we made a full length cDNA clone of RNA2. Restriction fragments (500–700 nt) were subcloned and sequenced. We observed a 98.6% homology between the sequence of Trk7-CMV RNA2 and the RNA2 of Q-CMV, the only strain of the same CMV subgroup whose RNA2 had previously been sequenced. The similarity was found to be 97% within the 5' and the 3' non coding regions (92 and 310 nt respectively) and 98% within the coding region. In the amino acid level, the replicase genes of the two strains were 95.8% identical. The highly conservative TG**T**NT and GDD motifs, which can often be found in the RNA polymerases of plant viruses, were also present in the central third of the protein. A homology of 70% was detected with the RNA2 of Fny-CMV belonging to the other subgroup of the cucumber mosaic virus.

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Cloning and integration of PPV CI gene into higher plants

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The cytoplasmic inclusion protein gene from a Hungarian isolate of plum pox potyvirus (PPV-SK68) was integrated into *Nicotiana benthamiana* L. plants.

The cytoplasmic inclusion protein of PPV is an RNA helicase and involved in the replication of the virus genome. The amino acid sequence of CI protein contains conserved nucleotide binding motifs (NTBM) in common with protein family of putative RNA helicases. Using the recombinant DNA techniques we have developed two gene constructions for plant transformation. The first contains the original coding sequence of

CI gene under the control of 35S promoter of CaMV and nos terminator signal, the second was mutated in the NTBM region. The recombinant plasmids with kanamycin resistance marker were mobilized into *Agrobacterium tumefaciens* and leaf disks of *Nicotiana benthamiana* L. were transformed. The transgenic plant lines were selected on kanamycin containing medium. The integrated viral genes were confirmed using polymerase chain reaction and the transcription was detected by Northern hybridization. The effects of integrated CI genes on the replication of plum pox virus and other related viruses are under investigation.

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Genetic mapping of bovine adenovirus type 10

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Bovine adenovirus type 10 (BAV-10) causes sporadic, fatal, haemorrhagic enterocolitis in cattle usually older than six months of age. Specific role of BAV-10 in such cases had been proven with the use of an in situ DNA hybridization method (J Clin Microbiol 34, 1270 1996). BAV-10 was originally placed into subgroup 2 BAVs, since it can be propagated on primary bovine testicle cells only, and has a genome of app. 30 kilo base pairs (Kb). In DNA hybridization experiments, however, BAV-10 did not cross react neither with subgroup 1, nor with subgroup 2 BAVs. The nuclear inclusion bodies produced by BAV-10 in cell culture are rather characteristic, and can be differentiated from inclusion bodies of subgroup 1 or 2 BAVs. Computer aided analysis of partial DNA sequences of BAV-10 also indicated that its phylogenetic distance is far from both subgroups. Our aim was to examine the genome organization and to determine the subgroup classification of BAV-10. First, detailed physical maps were constructed with several restriction enzymes, then the complete genome of BAV-10 was molecularly cloned. The DNA sequencing of the ends of the cloned viral fragments allowed us to identify the coded proteins, and determine the location of the genes on the genome. Our BAV-10 DNA sequence contingent is now over 10 Kb large. Physical and genetic maps are presented.

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Reactivation of Aujeszky's disease virus (SHV-I) from pigs

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Five pigs (average body mass: 60 kg)-derived from a "triple-free" (3F) farm were inoculated intranasally with Aujeszky's disease virus (SHV-1) of 10⁵ titre. Two pigs developed the nervous and respiratory symptoms typical of Aujeszky's disease and died on postinoculation day 7 and 8, respectively. In the three survivors, the clinical signs, the degree and duration of virus shedding (10-11 days, maximum titre: 10^{6.9}), and the results of serological tests indicated that the acute form of Aujeszky's disease had developed and passed off. After the infection experiment lasting 21 days, the clinical status of the pigs was recorded daily and nasal swab samples were taken at 3-day intervals to determine virus shedding.

The three surviving pigs were given corticosteroid treatment 116 days after inoculation. This resulted in the reactivation of SHV-I in all three animals. Virus shedding lasted for 10-14 days (with a maximum mean titre of 10^{2.9}). The virus content of the nasal secretion did not differ markedly from that of the pharyngeal secretion.

During the reactivation of infection the pigs showed mild signs of Aujeszky's disease (nasal discharge, conjunctivitis). Two pigs had died by the end of the reactivation treatment while the remaining animal was euthanized. The clinical signs leading to death could in all likelihood be attributed to the consequences of corticosteroid therapy.

The success of reactivation proved that the pigs had been latently infected during the 116-day period. This is supported by the negative results of virus isolation attempts from nasal swab samples collected regularly during the period of latency.

This model experiment constitutes an integral part of research efforts aimed at elucidating the underlying causes of the so-called single reactor phenomenon (the appearance of a single seropositive pig in herds free from infection).

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Transsynaptic viral labelling via Aujeszky's disease virus

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Transsynaptic tracing via neurotropic viruses is a powerful method to study the cytoarchitecture of the nervous system: functionally related, organ-specific pathways, neuronal circuits, chains of neurones from a peripheral organ to the brain can be visualized in this way. The injected viruses are taken up by the axon terminals via receptor-mediated endocytosis then transported via axoplasmic transport to the perikaryon, where the viral replication starts in the cell nucleus. The newly synthesized viruses are released by exocytosis then the virions infect the adjacent, synaptically connected neurones. In our experiments two Aujeszky's disease viruses (ADV) were applied: (1) Bartha's strain, an attenuated strain, used for vaccination of pigs, thus non-pathogenic, and (2) a recombinant virus, vE161ac strain. The recombinant vE161ac virus was generated by deleting of a 2976 bp DNA fragment which encompasses 1851 bp of the right UL component, the UL-US junction, the "a" element of internal repeat and a putative LAT promoter. *LacZ* gene of *Escherichia coli*, controlled by hCMV promoter/enhancer and containing a polyA signal of SV40, was ligated into HindIII site of pEh2 vector. The *lacZ* gene expression cassette was used to replace the deleted fragment, to produce recombinant virus vE161ac. The vE161ac strain showed similar pathogenicity to that of the wild type virus. The viruses were injected into the adrenal gland of rats, then 4 days later the spinal cord and the brain were processed for immune and enzyme histochemistry. Immunohistochemistry: the infected neurones were identified by immunoperoxidase staining of the monoclonal antibodies against the gI or gII viral proteins. In the case of recombinant vE161ac strain the result of virus infection manifested in expression of *lacZ* gene, e.g., β -galactosidase activity, detected by 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) as a substrate (Lojda Z.: *Histochemie* 23, 266 1970). Following injection of a small amount (2–10 μ l) of the ADV strains into the adrenal gland in a concentration of 2×10^8 PFU/ml, infected neurones were detected in the ipsilateral intermediolateral cell column and in the central nuclei (bilaterally) of the spinal cord. In addition, cells were labelled in the nucleus tractus solitarius, and in the ventral medulla oblongata, in the ventrolateral pons, in the hypothalamic paraventricular nucleus (in accordance with findings of Jansen et al. *Brain Res* 617, 103 1993). In the future we will extend our study to reveal the organ-specific neuronal circuits of other endocrine and exocrine organs.

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The physical state of human papillomavirus 16 in cancer of the uterine cervix

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Certain human papillomaviruses (HPV 16, 18 etc.) have an important role in the development of cervical cancer. HPV DNA is present in an episomal form in asymptomatic infections and benign lesions, while it is frequently integrated into the host cell genome in malignant lesions. In this study, the presence and physical state of HPV 16 DNA was investigated in the primary tumours and pelvic lymph nodes of 51 patients suffering from invasive cervical cancer, using Southern hybridization and polymerase chain reaction (PCR). The presence of HPV 16 DNA was demonstrated in 17 cases by Southern hybridization (10 cases harboured virus DNA in the primary tumours, 5 in the lymph nodes, and 2 in both locations). Virus DNA was present in an episomal form in 4 cases, integrated in the cell genome in 11 cases, and in both forms in 2 cases. Using a PCR method specific for the E7 transforming region of the virus, all the primary tumour samples and 88% (15/17) of the lymph node samples of Southern positive patients proved to be PCR positive. On the other hand, 41% (14/34) of the primary tumours and 24% (8/34) of the lymph nodes were PCR positive in Southern negative patients. Altogether, 61% (31/51) of the patients were positive with the above PCR method. Using another PCR method, the detectability of an intact E2 region was investigated in samples which were HPV 16 positive by Southern hybridization (usually, the E1-E2 region is disrupted and inactivated during integration). In those cases which harboured exclusively episomal or episomal and integrated virus DNA (6 cases), we managed to amplify the E2 region. On the contrary, only 36% (4/11) of the cases carrying integrated HPV 16 DNA were E2 PCR positive. Thus, there is correlation between integration and the detectability of the E2 region, but this PCR method alone is not suitable for the unambiguous determination of the physical state. Other PCR methods which can be used for this purpose have been presented.

VIROLOGY AND IMMUNOLOGY

H. ALLAKANY, K. FÁBIÁN, I. NÉMETH and L. STIPKOVITS

Immune chickens response in respiratory tract washings and serum of infected with *Mycoplasma gallisepticum* detected by immunoblot

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To improve diagnostic methods of mycoplasma infection it is important to know which proteins of infecting mycoplasma induce antibody response locally or in the serum. Therefore we have studied the immune response of chickens experimentally infected with *Mycoplasma gallisepticum* (MG). Two experiments were performed; the first one included 60 3-week-old chickens which were infected intra air sac with MG to follow the development of immune response locally in the respiratory tract and in serum against different MG proteins with different antibody classes along 3 weeks. The second experiment included 20 10 days old chickens infected by MG intra air sac. Two weeks later they were examined for the serological response by immunoblot.

We found IgM type of antibodies mainly against p64–67 and p56 in the respiratory tract and against p72, p64–67, p56 and p50 in the serum in weekly decreasing quantity. On the third week the IgM antibodies disappeared. Regarding IgG the reaction in the respiratory tract, on the first week it was against p120, p72 and p40 while in the serum IgG reacted to p150, p120, p72 and p40. On the second week we found antibodies against a lot of other proteins in the serum and also in the respiratory tract. The reaction IgA in the respiratory tract included antibodies to p72, p64–67, p56, p40, p31, p28, p26 and p24. Generally the reaction to IgA was negative in serum. Based on these data p72, p64–67 and p56 proteins are responsible for early IgA and IgM response. In sera the IgM and IgG antibodies reacted to much more proteins than in the respiratory tract.

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Serological response of broiler chickens infected with *Mycoplasma gallisepticum* after antibiotic treatment

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Antibiotic treatment may have an effect on the reliability of serological diagnostic methods. So we examined the immune response of broiler chickens infected with *Mycoplasma gallisepticum* (MG) and treated with 3 different antibiotics. We designed 5 groups with 60, 15 days old Arbor Acres broiler chicks in each. Four groups were infected with 0.2 ml of 10^7 CFU/ml MG strain 1226, intra airsac. Three infected groups were treated from the 1st to 4th day post infection with 50 mg/l drinking water (DW)

enrofloxacin (Baytril), 80 mg/l DW norfloxacin (Nortril) and 80 mg/l DW norfloxacin-nicotinate (Quin-Abic). One group was kept as infected untreated group and another as negative non-infected, untreated group. One, 2 and 3 weeks post infection, 20 chickens were slaughtered from each group. Serum plate agglutination and Western blot tests were employed to examine serological response. To control the infection, we checked the severity of pathological lesions (airsacculitis) and reisolation of mycoplasma.

From our examinations we can declare that in 20–100% of the control chickens had antibodies against all species-specific MG proteins. In spite of this, the Baytril treated group during the 3 weeks of experiment had antibodies against only p72, p64–p67 and p56 which appeared in 25% to 75% of the sera. In this group serum plate agglutination test positivity decreased along weeks post treatment from 90% to 25% of the chickens. The other two antibiotics did not decrease the serological response. The Baytril treated group had the pathological lesions and the reisolation rate as 1/20 and 1/5 respectively, when compared with infected non-treated group.

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Intertype specific epitope spectra of adenovirus hexons

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Three panels of monoclonal antibodies (MAbs) directed against the hexons of human 1 and 35, and bovine 2 serotypes recognized 22 epitopes on 21 hexon types, among them a genus and three type specific ones and 18 different bi- and multilateral intertype (IT) specific epitopes that grouped adenoviruses within the genus, independently from the subgenus they belong to. The distinction and marking of the different epitopes recognized by the MAbs were carried out by the determination of the composite cross-reactivity pattern, the titre and the correlation coefficient of all the 61 MAbs with purified preparation of 21 different hexon types representing all the six human subgenera, as well as different bovine and simian adenoviruses. The distinct epitopes were marked by two numbers referring the homologous hexon type to which the MAbs were directed and the serial number of the epitope specified by the different members of the given panel of the MAbs.

Considering that the type specific epitope could be present only on the homologous hexon type, the largest number of the different epitopes distinguishable by the MAbs used could be 20 on the homologous hexon and 19 on the heterologous ones. The number and distribution of the distinct specific epitopes on the different hexons was different, as expected. It was found that the total number of IT specific epitopes on the hexons varied between 2 and 18. The antigenic structure of the individual hexon types were characterized by the determination of their IT specific epitope spectrum. With the help of 18 epitopes specified by the MAb panels it was demonstrated that three epitope clusters could be distinguished, namely the types 4 and 19, types 8, 9, 9/13 and 10 as well as types 1, 2, 5 and 6, representatives of subgenera E-D, D and C, respectively. No clustering was found with human types 7, 12, 13, 18, 26, 27, 35 and 41, as well as with

bovine and simian adenovirus hexons studied. However, they displayed a closer or looser antigenic relationship among each other and to members of the epitope clusters. The degree of antigenic relationship could be expressed by the similarity/dissimilarity percentage calculated from the number of the identical and different epitopes present on any two given hexon types.

Based on these results, it seemed that the IT specific epitope cluster formation is independent from the grouping in subgenera. The well defined IT specific epitope cluster of subgenus C hexons is the only exception, because the identical IT specific epitopes of the four hexon types belong to the C subgenus.

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Detection of homologous DNA sequences in animal adenoviruses by polymerase chain reaction

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A primer pair originally designed for the universal detection of human adenovirus (I-IAV) serotypes of all subgenera was modified then tested and found feasible for the detection of different bovine, ovine, porcine, and canine adenovirus (BAV, OAV PAV, and CAV, respectively) serotypes. Apparently, in the examined viruses, parts of the DNA sequence coding for the basal part of the hexon protein are conserved enough for being applicable in polymerase chain reaction (PCR) as primers. Positive amplification could be obtained even from the so-called subgroup 2 BAVs, which viruses do not cross react with HAVs or subgroup 1 BAVs in Southern hybridization.

A. BÁCSI, J. SZABÓ, J. NEMES, J. KISS and F. D. TÓTH

Interactions between human cytomegalovirus and human T-cell leukaemia-lymphoma virus type I in CD4+ T-cell lines

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A high frequency of complications due to human cytomegalovirus (HCMV) have been observed in adult T-cell leukaemia (ATL). Since both HCMV and human T-cell leukaemia-lymphoma virus type I (HTLV-I) can infect CD4+ T-lymphocytes, interactions between these viruses may have an important role in the pathogenesis of ATL. The aim of our study was to investigate the replication of HCMV and HTLV-I in dually infected CD4+ T-cell lines. Expression of HCMV in the HTLV-I-carrying MT-2 and MT-4 cells was restricted at the level of immediate-early gene products, not depending on the HTLV-I producing ability of these cell lines. On the other hand, HTLV-I replication in MT-2

cells was markedly inhibited by superinfection with HCMV. HTLV-I could permissively infect H9 cells, whereas single infection of this cell line with HCMV did not result in release of infectious virus. Interestingly, coinfection of H9 cells with both viruses did not alter HTLV-I replication but mined HCMV infection from a nonpermissive to a permissive one. Dual infection of the same cellular targets was demonstrated by double immunofluorescence assay. These results show that HTLV-I and HCMV can interact during coinfection of CD4+ T cells to alter the expression of both viruses.

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Determination of HIV-1 subtypes in Hungary by synthetic peptides representing V3 loop of Env

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HIV divergency is based on the variation of the nucleic acid and amino acid sequences of the virus isolates originating from various geographical regions. HIV-1 strains have been classified so far into 9 different genetical subtypes ranging A to I according to the geographical origin and variability of the amino acid sequence of the immunodominant V3 loop of the gp120 (SU) envelope protein. For the determination of the dominant HIV-1 subtype inducing the majority of HIV infections in Hungary, a specific ELISA has been prepared with synthetic decapeptides as antigens characteristic to the env V3 loop of major classes of HIV-1 such as A, B, C and E.

DNA sequencing of HIV-1 *env* V3 loop encoding regions of field isolates was also determined with PCR amplification of a 211 nucleotide fragment of HIV-1 *env*. Alignment comparison was made with HXB-2, HIV-1 BRU(LAI) and HIV-1 NL43(NY5). More than 80% of the sera tested reacted exclusively with HIV-1 B antigens, 9.6% with A/B and 2.4% with A/C. Two sera reacted with none of the antigens used. No reactivity with HIV-1 E subtype has been found. Sera tested were collected between 1986 and 1996. Based on the sequencing data, showing high similarity (96.9%) of the Hungarian isolates to HIV-1 BRU(LAV) env V3 region as well as the serological analysis suggest that HIV-1 infection in Hungary has been induced dominantly (>80%) by HIV-1 subtype B.

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New optimization strategy for improving multi-variable ELISA assays

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To improve the sensitivity and minimizing background in ELISA assays different optimization methods have been applied to this field, but those cases when multi-variable setup needed to achieve this goal, the most frequently used method, so-called criss-cross dilution technique, is unsatisfactory. In this system only two factors can be considered in a given experiment, which means an unacceptable large number of experiments to catch the optimum condition. We used a statistical method which can deal with multi-variable setup and does not need a too large number of experiment. This variance analysis based method, Taguchi optimization, helped us to solve the problems what we encountered to detect ScFv antibodies (single chain fragment of variable antibodies) in ELISA assays. Exemplifying this ScFv assay system we introduce this Taguchi optimization method in the field of ELISA optimization in general.

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Amplification of the immune system of the newly born

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Ridge, Sarzotti and Fosthuber recently published on the updated concept of the accelerated ontogenic development of the not yet mature immune apparatus, the prevention of the tolerance induced against "not self" antigens in pre- and post-natal period. So, utilizing this principle in the immunization practice of both newly born and very young animals new vistas became opened up before extending the eradication of infectious diseases. Regarding the pragmatic consequences of the above published data, we started to analyse biometrically and to reinterpret (L. A. R. applying his own computer programme) our similar but forty year old experimental results, as yet unpublished. More than forty years ago, we immunized newly born and very young animals and babies against diphtheria with fair success. Historically: the late Prof. J. Lukács, Head of Pediatric Department of St. István Hospital alleged that the smallpox vaccine "teaches the not yet developed immune system to immunology". On this basis J. L. and L. R. started collaboration aimed at proving the validity of this theory, half a century ago.

Vaccines applied throughout the experiments represented the same batch of smallpox vaccine as well as the alum precipitated row (not purified) diphtheria toxoid (Glenny, Faragó and Lahiri) for immunization of newly born and very young guinea pigs and later, human babies. Three groups were formed who were immunized:

(i) Solely with smallpox vaccine. (ii) Groups immunized with diphtheria toxoid were divided to group of immunized (iia) who received only one shot of toxoid and (iib) who were immunized twice. Prior to diphtheria toxoid introduction, this (ii) group had not received any smallpox vaccine. (iii) The third group (consisting also of two subgroups) was vaccinated with smallpox prior the immunization with different doses of diphtheria toxoid.

The anti-diphtheria immunity was controlled with SCHICK reaction conversion test. The results unequivocally demonstrated that immunization was effective in the newly born presuming that smallpox vaccination was applied prior to the introduction of diphtheria toxoid. The authors claim a good congruity between present results and those achieved by them with lipopolysaccharides in immunologically impaired humans and animals, though one should not forget about the fifty year old achievements of Lahiri who isolated a "non-specific synergist factor" (presumably peptidoglycane) from the crude culture fluid of *Corynebacterium diphtheriae*. (That time the original and genetically not yet manipulated Park-Williams No. 8. strain had been applied. At present, however the genetically selected CN2000 strain is applied for diphtheria toxin production in submerged cultures). It has to be mentioned that using peptidoglycans L. R. and L. A. R. succeeded in increasing the "non specific resistance" in tumour infested animals as well as in applying the peptidoglycans as immuno-adjuvants. In their opinion these forty year old results – as reinvestigated and reinterpreted – do support the new concept on immune tolerance and the accelerated/forced development/amplification of immune potential.

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Programmed cell death of cultured immune cells induced by HIV-1 or FIV

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The process of programmed cell death (apoptosis) induced by HIV-1 or its feline model FIV is partially known, only. Few data are available on the inducing conditions and wide range sensitivity of different immune cells. To study these phenomena, suspension cultures of human and feline immune cells were infected at different m.o.i. by HIV-1 and FIV, respectively. Subsequently, growth, viability and morphology of cells were monitored at different time intervals. In serial samples, obtained between 12 h and 21 days, the 200 kb DNA ladder characteristic for apoptosis in agarose gel was studied. The ratio of dying cells was calculated by ELISA and flow cytometry. Possible direct role of HIV-1 gp120 was studied by mixing gp120 expressing Raji cells with human immune cells. It was established that, both HIV-1 infection and mixing with gp120⁺ Raji cells resulted in the immediate cease of growth, gradual decrease of viability of C8166 and Jurkat cells, but no effects were exerted on lymphoid H9 and monocytoid THP-1 cultures.

Apoptosis in these C8166 and Jurkat cells started at 12 h p.i., became maximal by day 3 and 4, and was completed by releasing DNA fragments into culture media on day 7. Interestingly, virus production reached its maximum around day 4. FIV infection of MBM cells resulted in rapid retardation of growth, gradual onset of decrease in viability. Morphological and biochemical signs of apoptosis of infected MBM cells were seen from day 3 and 4 onwards p.i., while FIV production became significant from day 7 and 8. FIV infection also induced immediate apoptosis in a few percent of cultured cells. Altogether, these data indicate that, apoptosis is not a direct cellular response to HIV-1 or FIV infection, but induction of cell death starts when non-soluble gp120 molecules attach to

cellular CD4 or CD9 receptors, respectively. Differences in the processes induced by HIV-1 and HV indicate that, early expression of some retroviral genes might directly modify the activity of cellular enzymes playing a role in the apoptotic process.

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**Comparison of antigen and mitogen induced in vitro
stimulation of sea bass (*Dicentrarchus labrax*) leukocytes**

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Methods for separation, maintenance and in vitro stimulation of sea bass (*Dicentrarchus labrax*) mixed leukocyte cultures were elaborated and a rapid tetrazolium (MTT) based colorimetric assay was adapted to test the reactivity of lymphoid cells obtained from head kidney, peripheral blood and spleen. Applying this fast and relatively simple method, kinetic and dose-response assays were performed using lectins (Con-A, PHA-P) and bacterial lipopolysaccharide (LPS) as superantigens, and inactivated *Pasteurella piscicida* bacterial suspension as specific antigen.

In the leukocyte microtitration assays the strongest stimulation was observed in the presence of PHA-P: proliferation activity of leukocytes obtained from peripheral blood and specially those of head kidney origin was significantly higher. LPS caused weaker but definitely significant proliferation in these cultures too. Con-A proved to be the least effective in the tests: mild (but statistically significant) stimulation was achieved only in peripheral blood lymphocyte cultures. *P. piscicida* antigen induced different responses in head kidney and blood leukocyte suspensions of fishes randomly chosen from aquacultures, presumably due to the different (though not recorded) anamnesis of the different fishes. Head kidney and blood lymphocytes from fishes experimentally infected and/or immunized with the same bacterial strain consequently produced elevated in vitro proliferation response, while spleen leukocytes did not show higher mitotic activity in the presence of the *P. piscicida* whole cell antigen.

B. SZABÓ, GY. VERESS, I. ANDIRKÓ, and L. GERGELY

The effect of tumour necrosis factor (TNF) on the cytotoxic activity of human papillomavirus (HPV) specific antibodies

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Seventy-two samples from the uterine cervix fluid (during and after the pregnant period) were tested for the presence of HPV-specific antibodies. Seventeen samples proved to be positive by ELISA using a synthetic peptide containing 24 amino acids of E7 antigen of HPV-16. Each of these samples and additional 14 ELISA negative cervical fluids proved to be cytotoxic for HPV-16 carrying CaSki cells in the presence of complement. The TNF-alpha pretreatment of target cells increased the cytotoxic activity of samples from $26.9 \pm 7.2\%$ to $39.2 \pm 9.2\%$. The cytotoxic reactivity of samples could be reduced to $10.8 \pm 8.2\%$ by pretreatment of samples using synthetic E7 peptide. In the majority of cases the amount of competitor and the reduction of cytotoxicity showed dose-response correlation. However, the TNF-induced increase of cytotoxic activity could be blocked less effectively by the competitor ($25.3 \pm 7.6\%$ residual activity). Similar results were obtained using sera of 25 cervical cancer patients.

Data suggest that the TNF treatment increases the cytotoxic activity of uterine cervix fluid and serum against HPV-16 carrying target cells, however this activity seems to be partially virus-specific. Studies on the potential biological role and possible targets of these reactions are going on in our laboratory.

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Complement-dependent enhancement/neutralization predicts clinical progression in advanced stage HIV-1 disease

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Previously we have reported on a strong correlation of the titres of complement-dependent enhancing antibodies with immunosuppression and disease in early and intermediate stage HIV-1 disease. The aim of the present study was to investigate if the same association can be found in patients in more advanced stage of HIV-1 infection. An enhancement/neutralization index (E/N) was calculated for monitoring serum samples. We have measured in Group 1 of patients a mean E/N less than 0.5 considered as neutralization in samples taken before and after the initiation of the antiviral treatment, whereas in Group 2 an E/N exceeding 0.5 considered as complement-dependent enhancement was found at least in one sample. Marked differences were observed between the two groups in the course of HIV-1 infection in favour of Group 1. In

contrast, neutralization was found in almost all cultures treated with the same sera without complement and no correlation between the course of HIV-1 disease and the extent of neutralization was observed. Our results suggest that measurement of enhancement and neutralization in serum samples in the presence of complement may provide data predicting responsiveness or unresponsiveness to antiviral therapy.

BACTERIOLOGY

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Frequency of resistance in clinical *Staphylococcus* isolates to narrow spectrum antibiotics with special reference to glycopeptides

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In the year 1995 the sensitivity of 1151 clinical isolates of *Staphylococcus aureus*, 951 *Staphylococcus epidermidis*, 77 *Staphylococcus saprophyticus*, 616 *Staphylococcus haemolyticus*, 548 *Staphylococcus hominis* and 387 isolates of other coagulase-negative *Staphylococcus* species to penicillin, oxacillin, macrolides, clindamycin, mupirocin, vancomycin and teicoplanin was examined on Mueller-Hinton agar with the standard disk diffusion test.

The frequencies of resistant isolates were 90.4–68.5% to penicillin, 12.6–55.0% to oxacillin, 19.7–69% to erythromycin, claritromycin and roxithromycin, 12.7–40.9% to josamycin and spiramycin, 13.3–46.5% to clindamycin. Because of the lack of a complete macrolide cross-resistance, in a routine test it seems to be worth to examine the sensitivity of the isolates to both erythromycin and josamycin in order to represent macrolides entirely. At the same time it would be interesting to study the cause and the clinical significance of the lack of cross-resistance. 4.8–14.2% of the isolates were resistant to mupirocin, 1.8–8.6% to vancomycin and 0.8–17.9% to teicoplanin. These data indicated that glycopeptide cross-resistance failed to appear because of either the lack of the expression of such a cross-resistance or the unsuitability of the disk method to detect it due to bad diffusion. For these reasons, and avoiding misinterpretations in the cases of such life-saving drugs, the determination of the minimal inhibitory concentration is proposed when a vancomycin and/or teicoplanin resistant isolate is seen by the disk method.

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Prevalence of *Chlamydia trachomatis* in a low-risk population

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Chlamydia trachomatis is the leading cause of nongonococcal urethritis and cervicitis in women. Because of the recent increases in the numbers of new cases and severe consequences, there is an urgent demand for the introduction of sensitive and specific rapid diagnostic methods.

A multicentre examination involving seven centres was sponsored by the Hungarian Ministry of Health and Welfare in order to provide a survey of *Chlamydia trachomatis* in the pregnant population. Between 01. 01. 1994 and 30. 06. 1995, 7495 women were tested (Fig. 1). The control group comprised healthy patients above 18 years. The seven centres were selected with regard to different aspects, from developed and less developed areas in the capital, two large provincial towns and various other provincial regions reflecting either an industrial or an agricultural background.

The nucleic acid hybridization method (PACE 2 Gen-Probe) was introduced in this low-risk population for the examination of *C. trachomatis*. In one centre, further two methods, antigen detection by ELISA (SYVA) and cultivation on the McCoy cell line (staining with SYVA FITC-labelled antichlamydia monoclonal antibody), were applied (Fig. 2).

International surveys and experience indicate that the proportion of the population threatened by *C. trachomatis* is above 10%. The overall average incidence of *C. trachomatis* cases in this low-risk pregnant population was 5.74%. The data from the different centres ranged between 1.6 and 9.7%. The chlamydia infected Hungarian pregnant population is below the critical 10%, but there is one Hungarian county, where the value is close to 10%. In this provincial, industrial area, the number of unmarried and divorced pregnant women in a low social economic situation is disproportionately high. For this disadvantaged population permanent *C. trachomatis* screening was suggested. In the other centres, screening for *C. trachomatis* and the treatment of positive cases and their partners was suggested for pregnant women with premature labour and preterm rupture of the membranes.

Fig. 1. Results of PACE 2 Gem-Probe method

Centre	No.	Pos.	%
Capital I.	364	28	7.69
Capital II.	715	20	2.79
Town I.	1157	42	3.65
Town II.	472	6	1.27
Countryside I.	2327	228	9.79
Countryside II.	1703	92	5.40
Countryside III.	757	12	1.58
Total	7495	428	5.74

Fig. 2. Results of comparative examinations

Method	Positive	Negative	N. D
Gen-Probe	53	1617	51
ELISA	18	934	769
Cultivation	7	462	1252

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Genetic diversity of *Proteus penneri*

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DNA of thirteen *Proteus penneri* strains of clinical origins derived from four European countries (Germany, 9; United Kingdom, 2; Turkey, 1; Hungary, 1) was examined for plasmid profile and by random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) method. All strains contained two large plasmids of about 60 and 70 kilobase pairs (kb) and four strains harboured a 6 kb plasmid, too. RAPD-PCR with primer CCGCAGCCAA resulted in strain-specific arrays of 4–15 bands in the 0.3 kb to 6 kb region for *P. penneri*. These primer bands of about 1.8 kb and 2.8 kb sizes in different intensities were characteristic for all *P. penneri* strains studied. With the exception of two strains the patterns differed from each other at least from one to several bands. Our results suggest a high level of DNA sequence diversity within this species. The RAPD method provides a fast, economical and reproducible means of epidemiological typing *P. penneri*.

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**Efficacy of vaccines against pasteurellosis of calves caused
by *Pasteurella haemolytica***

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The efficacy of a vaccine containing heat treated *Pasteurella haemolytica* serotype A1 culture and a vaccine produced of the extracted capsule material of the same strain were evaluated in a challenge experiment. Three groups each comprising 6 or 7, 2–3-week-old calves were formed. Animals in Group 1 were vaccinated with a vaccine containing inactivated, heat treated *P. haemolytica* A1 cells, calves of Group 2 received a capsule extract of the same strain. Both vaccines were adjuvated with aluminiumphosphate and administered subcutaneously twice, two weeks apart. Group 3 served as unvaccinated control. All the animals were challenged by intratracheal administration of a *P. haemolytica* serotype A1 strain on the 11th day after the second vaccination and sacrificed 6 days after challenge.

The clinical symptoms were less severe in the vaccinated animals, however no great differences could be seen between the efficacy of the two types of vaccines. Using a scoring system the average grade of pneumonia in the control animals was 2.6 while in both experimental groups it was 1.0. In the control animals 25% of the lung surface was affected by pneumonia but in the case of Group 1 and 2 calves these figures were 1.8% and 2.6% respectively. The difference was significant. The vaccination elevated the antibody titers against *P. haemolytica* A1 in the vaccinated animals when examined by the indirect haemagglutination test but no antibodies against leucotoxin could be detected. On the basis of the above results both vaccines proved to be effective in preventing pasteurellosis caused by *P. haemolytica* serotype A1 in young calves.

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**Inhibition of *Salmonella* colonization and invasion with
different methods in chicken model experiments**

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Among the methods of protection against salmonellosis in poultry are the use of probiotics, competitive exclusion (CE) flora or – in the latest time – live oral vaccines. The purpose of present experiments was to examine the efficiency of the above mentioned methods through the inhibition of colonization and invasion ability of *Salmonella* strains. In this project we examined the efficiency of these different methods against *S. enteritidis* and *S. kedougou*, and *S. typhimurium* strains, respectively, using

experimental infections in one-day-old chicken models. We came to the following conclusions: 1) Probiotics (Lactiferm or Ascogen) exert slight or not measurable protective effect alone. When we applied them together they were more efficient. 2) Antagonist flora (Broilact, Aviguard) had a better but inconsistent effect. 3) Live oral vaccine (Zoosaloral) was efficient in a homologous system (against *S. typhimurium* infection). Using a heterologous system (*S. enteritidis* challenge) the vaccine was still protective but less efficient.

S. BERNÁTH, GY. KUCSERA, I. KÁDÁR, GY. HORVÁTH and GY. MOROVJÁN

Study of protein patterns of *Erysipelothrix rhusiopathiae* strains by SDS-PAG electrophoresis and autoradiography

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Previously at the study of protein composition of *Erysipelothrix rhusiopathiae* strains, the protein fractions separated by sodium dodecyl sulfate-polyacrylamide (SDS-PAG) gel electrophoresis, were detected by staining method. In this work the strains were cultivated in synthetic broth containing S-35 methionine. The radiolabeling of proteins made possible to detect the protein fractions by autoradiography after SDS-PAG electrophoresis.

The protein patterns of the investigated five strains of subtype 1a, and seven strains of different types and subtypes (1b, 2a, 2b, 3, 12) showed characteristic variances. The simultaneous examination of the same strain gave identical protein pattern. The densitometric evaluation of autoradiographic patterns made possible the numerical comparison of protein patterns of strains. Evaluating the similarity and dissimilarity of protein fractions in strains subtype 1a, the strains gave 64.7–97.2% similarity. This value was between 40.0–76.5% at the strains of different types and subtypes. It can be concluded that this method is as suitable for examination of protein patterns of strains and for establishment of similarity as the staining method.

Z. KAPILLER, B. P. BÓZSIK, CS. BOGNÁR and B. LAKATOS

Lyme borreliosis in dogs

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Lyme borreliosis is a zoonotic disease, which is present worldwide in temperature zones. The causative agent is *Borrelia burgdorferi*, a spirochete named after its first describer. The disease is tick born, the most important vector in Europe and also in Hungary is *Ixodes ricinus*. Clinical disease has been described in humans and dogs most frequently. In humans clinical symptoms are categorized in three main groups. Early signs are migrating erythema at the site of tick bite and other places later on, sudden onset of

joint pain, lameness, inappetence and fever. In the second stage of the disease cardiological complications, glomerulonephrosis and neurological signs may develop. Severe chronic arthritis characterize the third phase of the disorder. These distinct signs are not always apparent and the stages can mix in individual cases. Lyme borreliosis is being regarded for this reason as a complex disease capable to mimic symptoms of other disorders. In dogs, the mentioned stages are not always present and the chronic form is quiet infrequently encountered. The authors describe the clinical symptoms here according to human medicine. They found lesions resembling erythema chronicum migrans, inappetence, lethargy, high fever and lymphadenopathy in one case, representing the signs of the first stage. They have also diagnosed *B. burgdorferi* infection in animals with severe bradycardia and with seizure activities (second stage). Therapy is by administration of β -lactam antibiotics or tetracyclines. The authors used clavulanate potentiated amoxicillin in doses of 50 mg/bw. po. bid for two weeks followed by 25 mg/bw. po. bid for another two weeks period. Affected animals (diagnosed having borellia infection serologically) which were treated early in the course of disease recovered uneventfully. The diagnosis of the disorder is mainly done serologically by PHA or more frequently by ELISA. Isolation of the pathogen is not a reliable method as it is often leading to false negative results. Preventive measures (bathing or spraying animals at risk with insecticides at areas harbouring *Ixodes ricinus*) should be initiated, and dog owners should be aware of the zoonotic potential of Lyme borreliosis.

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Experience on listeriosis in Hungary

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Informations connected with animal and human listeriosis have been collected since the fifties in our country. Yearly epidemiological data of human and animal listeriosis cases from the first cases up till the last ones of 1994 are presented. Incidence of healthy listeria excreters among human beings and animals as well as incidence of listeria in or on more hundred food samples (pork meat, poultry meat, minced meat, salami, smoked sausage, smoked dried ripened sausage, bulk tank milk, pasteurized milk, cacao milk, cheeses, cream, butter, semi-preserved meat products, ready to eat foods, confectioneries, vegetables) have been shown. Presence of listeria in the environment and on the instruments of food industrial factories are demonstrated. For cultivation and isolation of *Listeria* strains from samples different media (TNSA, LPM, Oxford, modified Holman, Fraser, UVM) and methods (cold enrichment, oblique light technique) have been used.

From the data it can be concluded that listeriosis is a rare human disease in Hungary. Only sporadic cases have been diagnosed but the possibility of epidemic events can be supposed. During the last some years the number of cases increased. Epidemiology of animal listeriosis is different. Late winter and early spring epidemics

are characteristic. Raw materials from animals as well as plants can be contaminated with listeria. When the processing technology and/or hygienic conditions are not satisfactory, these microorganisms may appear in the final products of food industry.

I. VERÉB, J. DEÁK, and E. NAGY

Comparison of different diagnostic methods for *Borrelia burgdorferi*

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Hungary

Several polysystemic diseases are caused by the spirochete *Borrelia burgdorferi*. The diseases have three stages: 1. Erythema chronicum migrans (ECM) appears on the site of the tick-bite or around it a few days after the infection. 2. Neurological and cardiological symptoms show up months after the tick-bite. 3. The late stage is characterized by the arthritis, which may appear weeks or even years after the primary infection.

The most reliable method for the diagnosis of the Lyme-disease is the isolation and identification of the *B. burgdorferi*. As this method is time-consuming, serological diagnostic methods, based on the detection of *B. burgdorferi* antibodies have been introduced. Between September 1, 1993 and March 31, 1996 we examined 1256 samples. Specific symptoms of Lyme-disease were diagnosed in 589 samples. The applied methods were the following: Biomedica ELISA, Pasteur ELISA, P39 Immunodot, Virotech ELISA, etc.

Seropositivity was approximately 12% (n=73) of the patients with the specific clinical symptoms: ECM correlated in 9%, neurological and ophthalmological symptoms in 14%, while arthritis in 13% with seropositivity. Statistical comparison between the different serological methods and symptoms showed that in the case of ECM and arthritis the Pasteur ELISA (n=8), in the case of neurological symptoms Biomedica ELISA (n=7), while in the case of ophthalmological symptoms Biomedica ELISA and Virotech ELISA (n=7) proved to be the most efficient. *B. burgdorferi* antibodies were detected with Biomedica ELISA in 29% of the cases, while in 22% of the examinations with Pasteur ELISA.

In the course of the past two and a half years we observed low correlation between clinical symptoms and laboratory results. Our observations agree with the international experiences, that is, we do not have at our disposal any perfect method for the laboratory diagnosis of the Lyme-borreliosis. Among the several tests of the different firms the results of Pasteur ELISA and Biomedica ELISA correlated most with the clinical symptoms; for the confirmation of the borderline samples the P39 Immunodot kit proved to be the most appropriate. A sensitive and specific screening method is needed for correct serological diagnostics, which should be completed with confirmatory methods like P39 ELISA or some other reliable Western-blot ones.

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Genetic diversity of *Helicobacter pylori* strains derived from the south transdanubian region

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Helicobacter pylori is a small Gram negative bacillus found in the stomach with active chronic gastritis and duodenal ulcers. DNA of 23 *H. pylori* strains derived from the South Transdanubian region was analysed by random amplified polymorphic DNA-PCR (RAPD-PCR) method. Primer CCGCAGCCAA resulted in strain-specific arrays of up to 11 bands in the 0.5 kilobase (kb) pairs to 5 kb size range. With this primer a 0.95 kb band was characteristic for 90% of the strains. Primer AACGCGCAAC resulted in up to 5 bands in the 0.4 kb to 4 kb size range. Our results reinforced the high level of DNA sequence diversity of *H. pylori*.

GY. J. MEZEY

Occurrence and antibiotic susceptibility of aerobic pathogenic bacteria in our diverse specimens in the years 1993–95

Children's Hospital of Buda, Budapest, Hungary

There is about 120 000 children belonging to our Institute's area. The diverse specimens of this circle are elaborated in our first-grade (no faecal and anaerobic examination) bacteriological laboratory. The senders are hospital departments, out-patients' departments, family doctors. The culture and identification has been made by the Lányi-method, the Gram-negative enteric rods identified by the Flow-system. The antibiotics susceptibility is tested by the diffusion method, using the Becton-Dickinson disks with its own dispenser. The data is processing partly manually, partly by a computer program.

In 1993 5766, in 1994 5822, in 1995 5810 mixed specimens has arrived, from them we tested the antibiotics susceptibility of 1697, 1723, and 1946 isolates, respectively.

This date was statistically analysed year-to-year. We obtained information about the occurrence and distribution of the pathogen bacteria, and their antibiotic susceptibility too. We presented the antibiotic susceptibility of the *Staphylococcus aureus*, *Escherichia coli* and some nosocomial infections causing bacteria, and also the most often isolated pathogens of the children having urinary tract infection.

The incidence of streptococci increased three-fold, the *Streptococcus pyogenes* four-fold during the observation time. The appearance of *Haemophilus* increased four-fold. The most frequent pathogens of nosocomial infections – *Acinetobacter*, *Serratia*, *Enterobacter*, *Pseudomonas* – appeared rarely in our specimens. The appearance of *Pseudomonas* decreased three-fold. The frequency of MRSA in our specimens was in 1993 1.5%, in 1994 2%, in 1995 3%. The proportion of penicillin resistant *Streptococcus pneumoniae* was in 1993 34%, in 1994 45%, in 1995 31%, erythromycin resistance was in 1993 31%, in 1994 37 %, in 1995 25%. The incidence of penicillin resistant *Enterococcus faecalis* was in 1994 22%, in 1995 14%, in 1995 33%, gentamicin resistance was in 1993 2%, in 1994 11%, in 1995 6%. No vancomycin resistant isolates were found.

BACTERIOLOGY MINISYMPOSIUM: NEW PATHOGENS – NEW ROLE OF OLD PATHOGENS

T. PÁL

Why now ? - Emerging infectious diseases at the end of the century

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Despite of the dramatic effects of the plagues of the past the achievements of epidemiology and microbiology, in the 19th, and in the first half of the 20th century, as well as the success of antibiotic therapy in the past fifty years lead to the false feeling of security. It appeared to many that the complete elimination, or at least control of the known infectious diseases is just a matter of further research, money, and time. However, recently this optimistic view had to be changed. During the past few decades the human race has had an impact on its natural and artificial environment to the extent never experienced before. This environment, however, is inevitably shared with the microbial world exhibiting an enormous adaptability. The increase of antibiotic resistant strains led to the reemergence of problems like tuberculosis, *Staphylococcus* or *Pneumococcus* infections, once thought to having been taken care of. Well known human pathogens gave rise to new syndromes while colonizing new niches (e.g. *Staphylococcal Toxic Shock Syndrome*), or their new variants cause new diseases (*Haemophilus influenzae* biogroup *aegyptius*). Altering the environment created or increased the possibility for certain microbes to "jump" from their natural hosts to man by extending the habitat of the host or that of a vector (e.g. Lyme disease, certain various mosquito-borne infections). Concomitantly, the industrialization of food production, the globalization of trade, the development of international traffic, the concentration of the population at many parts of the world, new cultural, behavioral patterns provide the possibilities of the very rapid

spread and globalization of infectious diseases. The economical, social and cultural factors leading to the emergence and spread of the new infectious diseases have been discussed together with our possibilities to meet the challenges they pose on us.

B. NAGY

**The role of receptors for toxins and adhesins
in pathogenesis of verotoxic *Escherichia coli* infections**

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Verotoxins and adhesins are heat labile proteins acting as important virulence factors for verotoxic *Escherichia coli* (VTEC). While toxic virulence attributes of VTEC are well known, our knowledge about their adhesins is quite limited. Present literary data and our experiences about receptors for toxins and for the known adhesins and about their role in the pathogenesis of VTEC induced diseases can be summarized as follows.

Verotoxins in general, have an A and several B subunits among which the subunit A (more exactly, its A1 compound) is responsible for toxic activity while subunit B contains the binding site of VT to glycolipid receptors in cell membranes. Within the above general feature, the main verotoxins (VT1 and VT2) and their variants (VT2e, VT2c, VT2d) have a wide range of biological differences, including receptors.

The receptor for VT1 and VT2 is globotriosyl ceramide (Gb3), while the receptor for VT2e is globotetraosyl ceramide (Gb4). Receptors on various types of host cells seem to determine differences in transport of verotoxin through the body, in target organs and, consequently, in the pathogenesis of VTEC infection. For instance, VT1 and VT2 bind to receptors of colonic epithelial cell membranes and to the mature red blood cells and to vascular endothelium at different rates, inducing haemorrhagic colitis, and/or haemolytic uraemic syndrome.

On the other hand, VT2e (the porcine edema toxin) has no binding to Gb3 but it binds to Gb4, present on porcine small blood vessel cells (especially on those of cerebellum, stomach and colon). This difference in receptors seems to be responsible for the vastly differing pathogenesis of VT1, VT2 in contrast to that of VT2e.

Regarding adhesin receptors of VTEC producing VT2e, there are strong indications about the presence of mannose containing glycoprotein receptors on the small intestinal microvillous membrane of weaned pigs but little or none on that of newborn pigs. This would explain why edema disease is not seen in newborn pigs but typically seen in weaned pigs. Adhesion of VT1 or VT2 producing VTEC seems to involve several factors including outer membrane proteins and attachment effacement (AE) gene functions.

One could hypothesize about further significance of age. related presence of toxin receptors in animals and man. Another area of hypotheses would be the therapeutic implications of receptor specificities.

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**Demonstration of verocytotoxins and their genes;
characterization of the *Escherichia coli* strains**

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The incidence of *Escherichia coli* causing haemorrhagic colitis (HC) or non-bloody enteritis in Hungary was studied using SLT I and SLT II gene probes and VT1, VT2 Verotox test. The presence of serotype O157:H-, O157:H7 and O157:HNT strains producing verocytotoxin causing HC or enteric cases occurred sporadically or in family outbreaks in Hungary was demonstrated using the Verotox F-test and specific DNA probes to SLT I and SLT II. Verocytotoxin production of other 28 serotypes of *E. coli* was proved by hybridization of DNA probes. In some cases strains containing SLT I and/or SLT II genes were demonstrated by hybridization but they were not expressed phenotypically. Multiplex PCR method was applied using specific primers to demonstrate the conservative sequences of the Shiga-like verocytotoxins (SLT I and SLT II) and the EHEC *eaeA* gene and the 60 MDa plasmid. Complex typing (sero-, phage-, colicin-typing and plasmid profile analysis) was carried out in *E. coli* serogroup O157 strains isolated from different geographical areas. The Hungarian phage typing method for *E. coli* strains used since 1978 was compared with the method elaborated in Canada in 1990. Out of the most frequent Canadian phage types (1, 4, 8, 31, 14) phage types 8, 31 and 14 were observed.

¹ZS. SZÉNÁSI, ²T. ENDO, ²K. YAGITA, and ¹E. NAGY

**The first isolation of presumably human pathogen
Naegleria fowleri in Hungary**

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"Free-living" amoebae of the genus *Naegleria* are found worldwide in different kinds of water. One of the species, *N. fowleri*, causes a serious human disease, the primary amoebic meningoencephalitis (PAM) leading usually to death. In most of the patients, the PAM have been related to previous bathing in geothermal pool or other water from which pathogenic *N. fowleri* was later isolated. Although cases of PAM have been reported from every continent, there have been no published cases in Hungary. Since swimming and bathing are very popular and wide-spread in Hungary, we decided to investigate the possible occurrence and frequency of the different amoebae, especially *N. fowleri* in the baths of Hungary. We isolated the first, presumably human pathogen *N. fowleri* from a swimming-pool in 1994. For specific identification, acid phosphatase and propionyl esterase activity of the water soluble protein extracts of the isolate were determined after a separation procedure by isoelectric focusing. The banding patterns

were compared to those obtained with *Naegleria* reference strains and isolates from other countries. A very probable human pathogenicity of our isolate has been ascertained since its isozyme profile is surprisingly similar to that of an isolate from the brain aspirate of a boy with PAM in Manila. In summary, we have evidence that the human pathogenic *N. fowleri* amoebae, causing PAM with mostly lethal outcome, can be found in Hungary, too; thus, in patients with meningoencephalitis, the possibility of a *N. fowleri* infection should be also considered.

É. BÁN

Changes in the role of Gram-positive bacteria in the infections of immunocompromised patients

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The significance of Gram-positive bacteria in the infections of immunocompromised patients has gradually changed in the last decade. Pathogens like enterococci, alpha haemolytic streptococci and *Streptococcus agalactiae* play new role in bacterial infections. *Enterococcus* has become the third most common pathogen in nosocomial infections while viridans streptococci have emerged as significant pathogens in the bacteraemia of neutropenic patients. *S. agalactiae* may cause sepsis not only in infants but also in immunocompromised adults. Coagulase negative staphylococci having been regarded traditionally as nonpathogenic bacteria are now frequent cause of polymer associated infections similarly to *Stomatococcus mucilaginosus* that has been almost unknown until recently. *Rhodococcus equi* as a new pathogen in medical microbiology causes serious pulmonary illness in patients with impaired cell immunity.

M. HEVESI

***Erwinia amylovora* is really a new pathogen?**

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“Fire blight” caused by *Erwinia amylovora* (Burrill) Winslow et al., is a disease that affects members of the family *Roseaceae* and causes considerable economic loss in pear- and apple-producing areas in North America, Western Europe (since 1957) and many other parts of the world. The disease was found – as a severe epidemic – on apple trees for the first time in the central part of Hungary in the spring of 1996. The pathogen was isolated from twigs and branches of the diseased trees and determined by biochemical, pathological and serological tests. No indications exist on the way of introduction of the disease to the country.

MYCOLOGY AND INDUSTRIAL MICROBIOLOGY

¹B. RIBÁR, ³A. BÁNRÉVI, ³GY. BERENCSI, and ^{1,2}M. SIPICZKI

Regulation of cytokinesis and separation in *Schizosaccharomyces pombe*

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Our understanding of the control of cytokinesis is limited in comparison with our knowledge of the controls over the initiation of S phase or mitosis. Study of genetically tractable systems such as *Schizosaccharomyces pombe* are a useful way to address this problem, since mutants defective in regulation of cytokinesis have been identified. The mutant *sep1*⁻ of *S. pombe* is morphologically defective in cell separation and forms long chains of unseparated cells.

To gain closer information about the *sep1*⁺ gene product and its biological activity, we cloned it. However, the mutant phenotype was not suitable for direct selection of the positive clones (*sep1* is not conditionally lethal), so the use of a conventional genomic library was not possible. Instead, we started with cosmid clones (30 kb) and P1 clones (70 kb) that overlap with the region to which we have mapped the gene (*sep1* was mapped: *sep1* is on chromosome II, close to *ade1* (0.94 cM)). The clones were provided by Dr. Lehrach (Imperial Cancer Research Fund, London, U.K.). We found a 2.4 kb positive DNA insert from the clones. We sequenced it by ALF. Express automatic sequencer (Pharmacia). We construct a disruptant allele and overexpress the *sep1*⁺ gene from a *pREP* overproducer plasmid.

The gene *sep1*⁺ was supposed to act in sister cell separation because it interacted with the late cytokinesis gene *cdc4*⁺. We found that the double mutant *sep1-lcdc4-8* formed cells with multiple nuclei when starved for nitrogen at permissive temperature. The *cdc4-8* has turned out to encode an EF-hand protein, similar to the myosin light chain, a component of the contractile ring formed prior to membrane furrowing (McCollum et al. J Cell Biol 130, 651 1995).

L. BÁNSZKY and A. MARÁZ

Induction and analysis of selenate-resistant mutants of *Schizosaccharomyces pombe*

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Selenate is a metabolic analogue of sulphate that is reduced to toxic selenite by the action of sulphate permease and reductase in *Aspergillus nidulans*. Selenate resistant mutants are unable to assimilate sulphate which can be divided into the complementation

groups of sB⁻ and sC⁻. Mutants belonging to sC⁻ showed chromate resistance at the same time. Selenate resistant mutants of *Schizosaccharomyces pombe* were induced in our laboratory at high frequency which were unable to utilize sulphate and thiosulphate as a sole sulphur source, while the utilization of organic sulphur source as methionine and cysteine was not influenced. Selenate resistant mutants could not be grouped according to their chromate sensitivity because wild type (selenate sensitive) strains showed a high degree of chromate tolerance. Selenate resistant mutants were proved to belong to the same complementation group according to the complementation analysis performed. Hydrogen sulphide production of the mutants was considerably reduced in the presence of anorganic N-sources while there was no change in organic N-sources.

ZS. FEHÉR

Common subunits of RNA polymerases in the fission yeast *Schizosaccharomyces pombe*

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Yeast RNA polymerases I, II and III are organized around a common core of subunits related to the bacterial core enzyme ($\beta'\beta\alpha_2$) and share five small subunits (ABC27, ABC23, ABC 14.5, ABC 10 α and ABC 10 β). All these common subunits are essential for growth, but their function in transcription is still elusive. Our aim is to clone some of these genes from the fission yeast to contribute to the understanding of the role of common subunits in transcription. Using sequence data of cloned yeast (*Saccharomyces cerevisiae*) and human genes, PCR primers were designed to amplify fragments of ABC10 β (Sp10 β) and ABC27 (Sp5). Experiments to clone these genes from *S. pombe* are under way.

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Growth characteristics and chromium accumulation of wild-type and chromium sensitive *Schizosaccharomyces pombe* strains

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Schizosaccharomyces pombe lys1-131h⁺ (MCCP 116) and leu1-32h⁻ (MCCP 119) auxotroph strains were treated with N-methyl-N'-nitro-N-nitroso-guanidine, and chromium sensitive mutants were isolated.

The minimal inhibitory concentration of chromate, given as K₂Cr₂O₇ after sterilization into YES agar (0.5% yeast extract, 3% glucose plus 100 mg/litre adenine,

histidine, leucine, uracil and lysine) was 150 μM in the case of wild type strains, and 75 μM in the case of the mutants.

Growth parameters in shaken cultures at different chromium content were followed by measurement of OD_{660} . The growth was characterized between 10–500 μM chromium (VI) contents in YES media. The growth curves showed that the chromium sensitive strains start to grow after a long lag period. The generation times and the lag periods, characterized well the differences between the wild type and the mutant strains.

The accumulation studies were carried out in CHEMUP bioreactor. The changes of pH, pO_2 and the biomass of the wild type strain were determined at different chromium content. The dry biomass was 1.92 g/l in the control; 1.5 g/l at 25 μM ; 1.42 g/l at 37.5 μM and 0.9 g/l at 75 μM Cr(VI) after 44 h. The chromium content in the biomass was determined with atomic absorption spectrometry using graphite furnace. After two kinds of preparation method, the wet biomass was treated with HNO_3 for "total chromium" and with NH_4OH for "organic bound chromium" analysis. At 75 μM Cr(VI) in the YES media, the cells of the wild type strain could accumulate 11.5 mg chromium/g dry biomass after 72 h.

T. DEÁK

Yeasts as emerging pathogens

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Beside the traditional humanpathogenic yeasts, such as *Candida albicans* and *Cryptococcus neoformans*, the number of yeast species isolated from clinical cases is increasing. Among these emerging yeasts are *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and some 30 other species. These opportunistic pathogens mostly occur with some underlying diseases (cancer, HIV) and also as nosocomial cases particularly with long intravenous catheters. The emergence of yeast infections is due to the use of antibacterial antibiotics and antimycotics as well, and can also be attributed to the improved methods of identification. Many yeasts isolated from clinical samples are among the most common foodborne species such as *Saccharomyces cerevisiae*, *Debaryomyces hansenii*, *Pichia anomala*, *Issatchenkia orientalis*, in turn, pathogenic yeasts are more frequently isolated from various foods. Emerging yeast pathogens should attract more attention of both clinical and food microbiologists.

¹Á. SVEICZER, ¹B. NOVÁK, and ²M. MITCHISON

Genetic background of size control in fission yeast

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In growing cultures to maintain homeostasis cells co-ordinate growth and division by size control mechanisms: they are able to execute some cell cycle events only if they have reached a critical size. In the fission yeast *Schizosaccharomyces pombe* size control acts in G2-phase controlling the onset of mitosis, which can be examined simply by time-lapse microphotography. Cells which are large at birth have shorter cycle times than cells which are small at birth. As a consequence larger cells grow less during their cycles than smaller ones. The negative correlation between length extension and birth length shows the existence of size control.

The level of mitotic size control and as a consequence length at division are influenced by some activators (Cdc25, Cdc2, Nim1) and an inhibitor (Wee1). We wished to determine which of these regulator proteins has (or have) an essential role in size control. Therefore we have examined the presence or absence of mitotic size control in different cell cycle mutants. We have found existing size control in *cdc2* and *nim1* mutants, so these genes are not essential. On the other hand, size control is absent in the double mutant $\Delta cdc25wee1.50$, so at least one of these genes is essential. Since size control is present in the double mutant $\Delta cdc25cdc2.3w$, we have proved that *cdc25* is not essential, only exclusively *wee1*. *wee1* gene encodes a 107 kD tyrosine kinase, which is a dose dependent inhibitor of mitosis. We suppose that mitotic size control acts through dilution of Wee1 protein during cell growth.

Because of lacking mitotic size control, *wee1* mutant cells are small. Even in these mutants we could prove the existence of size control that is as strong as in wild type cells. This size control acts at Start event in G1-phase, controlling the onset of DNA replication. On the other hand, this size control is absent in the double mutant $\Delta rum1wee1.50$. This means that the essential regulator of size control at Start is the cyclin dependent kinase inhibitor encoded by the *rum1* gene.

¹GY. VÉGH, ²T. ZAGORC, and ¹A. MARÁZ

Molecular analysis of yeasts isolated from the region Tokaj wine

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Certain strains belonging to the genera of *Saccharomyces* produce killer toxin proteins (zymocin) which impedes the reproduction of other sensitive yeast strains. Killer strains among yeasts isolated from fermenting wines of Tokaj Wine Region were selected. One part of these strains showed killer activity between pH 4–5 while the rest was active only at very narrow interval (pH 4).

Nucleic acids were isolated from 32 selected killer strains then separated with gel electrophoresis. In 26 strains the L and M dsRNS typical of killer strains were revealed while in 6 strains none of these fractions was present. In the latter ones, probably the killer gene(s) are coded on the chromosome. The size of L dsRNS were the same in every samples (4.7 kbp) while the size of M dsRNS varied from 1.8 to 2.1 kbp.

Electrophoretic karyotyping of strains indicated that 29 from 32 samples belonged into *Saccharomyces sensu stricto* group. With the use of six non-specific oligonucleotide primers RAPD-PCR analysis was also carried out proving that the isolates could be classified as *S. cerevisiae*.

M. KALYAN, G. PÉTER, D. DLAUCHY, and T. DEÁK

Characterization of some industrial yeasts: comparison of some currently available methods

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Nine yeast strains were isolated from wine, beer and baker yeast, all of which originated from India. The strains were subjected to classical identification tests according to Kreger-van Rij (1984), and where necessary, tested for presence of active fructose transport (Rodrigues et al. 1990). The strains were identified based on the results of the above tests and key of Kreger-van Rij (1984), as *Saccharomyces cerevisiae* (7), *Pichia fabianni* (1) and *Issatchenkia orientalis* (1).

Although classical microbiological tests were useful in identifying the above strains; these tests cannot readily distinguish and identify beyond doubt the four taxons of the industrially important yeast group – *Saccharomyces sensu stricto*. Hence the present study was undertaken, with a view to a) re-identify the above strains by molecular technic, in order to check the results of classical method; b) to evaluate the applicability of different molecular methods in identifying the above yeast strains. The yeast strains were subjected to the following molecular characterization: molar base composition

analysis, electrophoretic karyotyping, Random Amplification of Polymorphic DNA (RAPD-PCR) analysis; and estimation of nDNA/nDNA reassociation. The results of the molecular characterization corroborates the results obtained by classical method. A comparison of sensitivity of different molecular methods to differentiate the isolated yeast, is also discussed.

¹T. M. SAAD EL-DIN, ¹J. REZESSY-SZABÓ, ²L. DENCŐ, and ¹Á. HOSCHKE

**Effect of environmental parameters on the expression
of *LacZ* gene governed by *ADHI* promoter
in *Saccharomyces cerevisiae***

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There is significant interest in the use of microorganisms for the production of heterologous proteins. Optimization of the production and large scale industrial application of recombinant DNA mediated systems require detailed information about genetic and physiological background.

To define the effect of the environmental parameters on the expression of gene controlled by *ADHI* promoter the complete promoter was fused to the *LacZ* gene of *Escherichia coli* encoding the β -galactosidase enzyme in Yep-type plasmid and a *Saccharomyces cerevisiae* strain was transformed by this plasmid. However, the *ADHI* promoter is constitutive, but it can be regulated. Our results indicate, that the β -galactosidase production of *S. cerevisiae* harbouring the recombinant plasmid has reached higher value on fermentable sugars than in case of non-fermentable carbon sources. Furthermore the maximum production was detected in the presence of glucose as sole carbon source. Increasing the concentration of glucose up to 2% the β -galactosidase production has grown. Changing the ethanol content of the medium failed to effect significantly the enzyme production. Moreover we have investigated the influence of the temperature and the pH. These results can be contributed to the maximization of the production of heterologous protein.

T. JAKAB and B. LENKEY

**The effect of fluconazole in vitro on the growth
of *Candida albicans* under anaerobic condition**

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Candida albicans is a dimorphic yeast, which can grow either as an ellipsoidal bud (often referred to as the blastospore or the yeast form) or in a filamentous form producing pseudohyphae or true septate hyphae. *C. albicans* may be frequently isolated from the

mouth and/or vagina and yeasts including this dimorphic yeast are inhabitants of the gastrointestinal tract of most healthy individuals. The gastrointestinal tract serves as normal reservoir from which the yeasts can penetrate the gut wall and disseminate via lymph and blood vessels. Colonization by *C. albicans* is usually limited. It is well known that in patients with mycotic complications the *Candida* can be recovered from urine, sputum and faeces samples too, and its counts range from 10^3 to 10^8 CFU ml⁻¹. We used an in vitro model under anaerobic condition (a mixture of N₂ and CO₂) that is adapted to simulate the gastrointestinal tract to determine the growth of *C. albicans* and the effect of fluconazole on the growth of *C. albicans*.

Two different fluconazole-susceptible strains of *C. albicans* were used. Stock cultures of *C. albicans* were maintained in Sabouraud dextrose agar slopes at 4 °C, and were transferred monthly. Two to three standard loopfuls of these cultures were inoculated into 10 ml of YPG medium (1% yeast extract, 2% mycological peptone and 2% glucose) and incubated for 18 h at 37 °C. Five to seven ml of final yeast suspension were transferred into 560 ml of YPG medium and divided into 8 equal pans. Incubation was carried out in 300 ml Erlenmeyer flasks for 24 h at 37 °C with orbital shaking at 150 rpm. Four of them were incubated aerobically and the another four were cultured anaerobically with or without added fluconazole (the final concentrations of fluconazole were 1.5 µg.ml⁻¹, 3.0 µg.ml⁻¹, 4.5 µg.ml⁻¹ and 0 µg.ml⁻¹ (as control). The turbidity of the yeast growth in each population in terms of A₆₀₀ measured by spectrophotometer. The growth of yeast strains and the inhibition of their growth by fluconazole were different under aerobic or anaerobic conditions. The growth of the fluconazole sensitive strain was inhibited in 50% while the fluconazole resistant strain was only inhibited in 15% under anaerobic condition without fluconazole. Microscopic examination revealed a different kind of growth under aerobic and anaerobic conditions.

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Vegetative compatibility grouping and identification of molecular markers in *Fusarium poae*

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Fusarium poae is a world-wide distributed secondary plant pathogen invading small-grain cereals and pasture grasses. This fungus causes considerable additional damage of food and fodder crops by its toxic metabolites that may accumulate in high concentrations under adverse environmental conditions. Not much is known about the genetic diversity within this strictly asexual fungus which probably lacks an efficient mitotic recombination mechanism as well.

Vegetative compatibility and random amplification of polymorphic DNA (RAPD) were used for assessing genetic interrelationships among 55 geographically different strains originating from Australia, Europe, Japan, New Zealand, North America and South Africa. Vegetative compatibility of individual strains was demonstrated with heterokaryon

formation between complementary nitrate-nonutilizing (*nit*) mutants, while RAPD markers were identified using the synthetic primers OPE-03, OPE-07, OPE-15, OPE-18 (Operon Technologies).

Thirty-five strains of *F. poae* could be placed into 13 vegetatively compatible groups (VCGs), the remaining 20 strains were not compatible with any other isolates. Four bridge-strains were found, they proved to be compatible with isolates that belonged to different VCGs, enabled thus tracing inter-VCG affinities. A complex interaction was observed between VCGs and geographic origins. The majority of the Hungarian isolates formed VCGs, distinct from other strains, and similar aggregation tendencies were observed in certain isolates from South Africa, Japan and New Zealand indicating the predominance of genetically non-interbreeding populations in these regions. On the other hand, a strikingly heterogeneous sample was received from North America. Nevertheless, the subdivision of *F. poae* into geographically isolated sub-groups is not reasonable because limited compatible reactions occurred between certain strains originating from different continents, like Europe and North-America or Africa and New Zealand.

A total of 90 RAPD markers were identified in the 55 strains. The data were tabulated in a matrix from which a phenogram was constructed by means of cluster analysis. The RAPD-patterns were highly correlated to vegetative compatibility groups and promoted the affiliation of single-member isolates. The phenogram suggests, that in spite of the wide-spread vegetative incompatibility and the lack of meiosis in *F. poae* this fungus is a distinct genetic species and shows relatively little intraspecific divergence. The high number of VCGs indicate, that pathogen specialization has not been evolved in this fungus.

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Efficacy of orungal (itraconazole) therapy in onychomycosis

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The pulse therapy with Orungal, according to comparative clinical studies, proved to be more successful for fungal nail infections than continuous treatments [1–3]. This therapeutical method is considered to be effective because of the recidivation is decreased by the drug, the therapy is not accompanied by side effects and the using of the medicine is economical. We with special regard to these observations, studied the pulse therapy with Orungal for onychomycotic patients. Twenty-one patients were joined into the therapy: onychomycosis took place on fingernails in 13 cases, and on the toenails in 12, respectively. In positive cultures *Candida albicans* occurred in 7, other yeasts in 10, *Trichophyton rubrum* in 7 and finally *Aspergillus niger* in one case.

During pulse therapy patients received 200 mg Orungal, twice a day, for a week. After three weeks' pause the cure was repeated twice or thrice in case in fingernail infections and thrice or four times in toenail infections, respectively. The status of one

target nail was controlled at all patients. After the therapy, the clinical cure and marked improvement were observed in 8% and 88% of patients, respectively. The mycological cure was 84%. Half year later, the repeated examinations displayed a clinical cure of 52%, marked improvement of 48% and mycological cure of 96%. Side effects, such as hyperaemic exanthema and increased hepatic enzyme activity (gamma-GT, GOT) were observed in one and four cases, respectively. The pulse therapy applied in the treatment of onychomycosis proved to be successful and was recommended to wide-range introduction into the therapeutical practice.

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¹J. VARGA, ¹E. RINYU, ¹É. KEVEI, ¹F. KEVEI, ¹ZS. HAMARI, and ²J. TÉREN

Ochratoxin production by *Aspergillus* species

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Ochratoxin production was tested in 330 strains representing most species in sections *Nigri*, *Fumigati*, *Circumdati*, *Candidi* and *Wentii* of the *Aspergillus* genus by using different approaches, including immunochemical methods based on an enzyme-linked immunosorbent assay (ELISA), thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). The monoclonal antibody-based ELISA technique was successfully used to screen ochratoxin A production in the black *Aspergilli*. Ochratoxin A (OA) was detected in the culture filtrates of one *A. foetidus* (*A. niger*), one *A. awamori* (*A. niger*), one *A. niger* and five *A. carbonarius* strains [1]. OA was also detected in *A. ochraceus*, *A. alliaceus*, *A. sclerotiorum*, *A. sulphureus*, *A. albertensis*, *A. auricomus* and *A. wentii* strains. This is the first report of production of OA in the three latter species. OA production of these species was confirmed by thin layer chromatography and by high performance liquid chromatography. The ELISA-based method might be useful for the identification of weak ochratoxin A producers, and for taxonomic purposes.

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B. TÓTH, ZS. HAMARI, R. BARNA, J. VARGA, and F. KEVEI

Phenotypic and molecular characterization of black wild type *Aspergillus* isolates

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One hundred and seventy-five black *Aspergillus* field isolates collected from stored onion bulbs, green coffee beans and soil samples from different parts of the world were compared as regards their carbon source utilization spectra, ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) restriction patterns, and mycovirus contents. Based on the carbon source assimilation spectra of the isolates, most of them could readily be allocated to one of the *A. niger* aggregate, *A. japonicus* or *A. carbonarius* species [1, 2]. Some strains exhibited unique carbon source utilization pattern, which observation underlines the usefulness of molecular methods in the assignment of field isolates to black *Aspergillus* species.

Based on the *Sma*I digested rDNA and *Hae*III-*Bgl*II generated mtDNA profiles, most isolates belonging to the *A. niger* aggregate could be allocated to one of the earlier described subgroups of the *A. niger* and *A. tubingensis* species [3, 4]. Some isolates displayed new mtDNA patterns (possible new subgroups). One strain isolated from a Brazilian soil sample represents a new mtDNA subgroup within mtDNA type 3, which was described earlier as possibly representing a subspecies of the *A. niger* species [4]. Sixteen *A. japonicus* and three *A. carbonarius* isolates exhibited the same types of rDNAs and mtDNAs as described earlier. Mycoviruses were detected in about 15% of the isolates examined, which is comparable with the frequency found earlier in other black *Aspergillus* field isolates [5]. An earlier undescribed new dsRNA profile was also detected in a strain isolated from Panaman soil.

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ZS. HAMARI, I. PFEIFFER, B. TÓTH and F. KEVEI

Physical maps of polymorphic mtDNAs in *Aspergillus carbonarius* strains

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Aspergillus carbonarius (Thom) is possibly the most distinct member of black *Aspergilli* exhibiting some unique characters. Studying the molecular polymorphism of this species among thirteen examined strains two main mitochondrial DNA (mtDNA) groups could be distinguished (designated type 1 and type 2 respectively) [1]. These groups correspond to their nuclear rDNA types. The mtDNA type 1 could be divided into two subgroups (labelled type 1a and type 1b respectively) based on slight alterations of

their RFLP patterns. Despite of the significant similarities of their restriction patterns 1000 base pairs differences were estimated between them (61.3 kb and 62.3 kb, respectively). Size of mtDNA type 2 proved to be 42.7 kb. The size difference between two main mtDNA type is 20.5 kb.

For interpretation of these considerable differences between three groups an attempt was made to construct their more detailed restriction maps. Isolated mtDNA was digested by *EcoRI* and the fragments were separated by electrophoresis and either were cloned into pBluescript KS plasmid or were redigested by several restriction enzymes, such as *XhoI*; *KpnI*; *BamHI*; *PstI*. On the basis of these data linearized physical maps of both subtypes (1a and 1b) and mtDNA type 2 were developed. Digoxigenin-labelled hybridization probes were applied for identifying the possible positions of certain regions.

ZS. ANTAL, L. MANCZINGER, and L. FERENCZY

**Investigation of integrative and autonomously replicating
plasmids in a *Trichoderma harzianum* strain**

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Sixteen arginine auxotrophic mutants were isolated from a mycoparasitic strain of *Trichoderma harzianum* AH-48. Ten of these mutants grew in the presence of citrulline, suggesting that the mutation took place in the *argB* gene. These mutants could be arranged into two groups based on the heterokaryon test. One mutant from each class was transformed with the integrative pILJ16 [1] and the autonomously replicating ARp1 [2] plasmids, carrying the *Aspergillus nidulans argB* gene, and both the *Aspergillus nidulans argB* gene and the *Aspergillus nidulans* AMA1 sequence, respectively. The transformation frequencies were 1 to 4 transformants per µg DNA in both cases. The presence of the AMA1 sequence had no significant effect on the transformation frequencies achieved. The transmission of the integrative pILJ16 plasmid into the conidia was inhibited. The presence of the plasmid-copies in the transformants was investigated by hybridization with digoxigenin labelled DNA.

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Application of carbon utilization tests in *Mucor* systematics

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Mucor is an important fungal genus because of its ubiquity in nature and its growing industrial importance. The accepted, comprehensive classification of this genus was based mainly on morphological observations and mating experiments. However, such approaches are appreciably hampered by the lack of good morphological markers and by the great variability of the available characteristics.

The aim of the present work was to study the applicability of physiological markers for the characterization of *Mucor* isolates. As part of this, carbon utilization patterns and the presence or absence of various exoenzyme activities were used to obtain characters for numerical analysis. Twenty-seven swains representing ten *Mucor* species were assayed for their ability to utilize various substrates as carbon sources. They were also examined for their exoenzyme activities by means of the API-ZYM microtest system. Cluster analysis of the resulting data revealed a loose correlation between these physiological patterns and the species designation of the isolates. Though different strains of the same species often vary in their substrate utilization abilities, the value of this approach for the provision of complementary data for the characterization of *Mucor* isolates has been demonstrated.

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Detection of double-stranded RNA molecules in different *Mucor* species

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During recent decades, double-stranded ribonucleic acid (dsRNA) molecules have been found in a wide variety of phylogenetically diverse fungi. In a majority of the cases, these discrete high molecular weight molecules were considered to be indicators of the presence of virus-like particles in the cells [1]. In this respect, fungal species belonging to the *Zygomycetes* are rarely screened; there is only one report on the presence of dsRNA molecules in a *Mucor* species [2].

The presence of dsRNA elements was examined in 123 isolates representing 18 *Mucor* species. These genetic elements were found to be present in 6 strains: 1 *M. aligarensis*, 1 *M. hiemalis*, 2 *M. corticolus*, 1 *M. mucedo* and 1 *M. ramannianus*. Electrophoretic separation of the nucleic acids revealed 4 different RNA patterns, with 1 to 5 discrete dsRNA bands. The molecular weights corresponding to these bands were $1.42\text{--}4.15 \times 10^6$ D. Electron microscopic examination also revealed the presence of small isometric particles in all 6 isolates.

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¹L. KARAFFA, ²J. KOZMA, and ¹A. SZENTIRMAI

Regulation of the alternative respiration in *Acremonium chrysogenum* by phosphate and the carbon source

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In our previous studies it was established that the filamentous mould *Acremonium chrysogenum* possesses a cyanide-resistant alternative respiration in addition to the cytochrome one. Among factors regulating respiration, the oxidized and reduced NAD-content of cells, the degree of ADP-limitation, the effect of the oxygen tension, oxygen concentration and growth rate was examined. In this study, the effect of phosphate concentration and of the quality of carbon source on the alternative respiration of cells was investigated. The following results were obtained:

1) When cultures grown in complete medium were washed into minimal medium with different phosphate concentrations, the respiration of cells were found proportional to the amount of phosphate present. Above a critical value (about 5 g/l) respiration was not limited by the phosphate at all.

2) In spite of the uninhibited respiration, the alternative respiration was inversely proportional to the phosphate concentration. Above the 5 g/l phosphate concentration, the differences started to decrease, the curve described this effect had a saturating character.

3) Cells transplanted in the exponential phase required more phosphate compared to the ones transplanted in the stationer phase. However, in the case of alternative respiration, the results were just the opposite.

4) Cells grown in complete medium and transplanted into minimal ones containing different carbon sources exhibited different capacity of alternative respiration. The results, in the percentage of uninhibited respiration were the following in decreasing order: pyruvate – soybean oil – glucose – malate – citrate – succinate. In the case of pyruvate, a complete cyanide-resistance was obtained, about 60% was found on soybean oil, while the other results were between 20 and 30%.

Finally it was concluded, that the alternative respiration of cells was inversely proportional to the concentration of phosphate. This result fits the fact, that the alternative respiration produces one third ATP only, therefore the electron transport by this route needs less phosphate. However, the capacity values measured on different carbon sources fail to justify the energy overflow hypothesis as it were not the glucose and the soybean oil, where the highest alternative respiration values were found.

T EMRI, I. PÓCSI and A. SZENTIRMAI

The role of glutathione in the protection against oxidative stress caused by peroxides in *Penicillium chrysogenum*

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In aerobic organisms during the reduction of molecular oxygen active oxygen species (superoxide anion radical, hydrogen peroxide, hydroxyl radical) are generated which attack almost all cell components. Against such active oxygens, cells have some defensive mechanisms including superoxide dismutase, catalase, peroxidases, and antioxidants such as ascorbic acid, tocopherol, β -carotene or glutathione. Here we show the changes in the glutathione metabolism during the oxidative stress caused by hydrogen peroxide and cumene hydroperoxide added to the culture media of *Penicillium chrysogenum*.

In the presence of peroxides the intracellular peroxide and GSSG pool increased markedly but while in the case of hydrogen peroxide (40–80 nM) no change or a small increase in the OSH pool was observed cumene hydroperoxide (2–4 nM) could deplete it completely. The effect of both peroxides on the specific enzyme activities was very similar. Specific glutathione peroxidase (GP_x), glutathione reductase (GR), γ -glutamyltranspeptidase (γ GT) and glutathione producing activities increased meanwhile no changes in the specific glucose-6-phosphate dehydrogenase, catalase and glutathione-S-transferase activities could be observed.

These findings indicated that, in contrast to numerous other microorganisms, *P. chrysogenum* produced GP_x , which played an important role in the protection against peroxides. To restore the glutathione status cells increased both the production and the regeneration of the reduced GSH form but surprisingly there was no increase in the activity of the pentose-phosphate shunt, which is thought to supply the NADPH requirement of the GSSG reduction. The role of the increased γ GT level has not been yet elucidated but it could have taken part in the elimination of either GSSG or toxic agents priced during the lipid peroxidation. The different effect of hydrogen peroxide and cumene hydroperoxide on the intracellular GSH and peroxide pools could be explained well with the high catalase activity found in the mycelia. Obviously, catalase was more active against hydrogen peroxide than with cumene hydroperoxide.

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Connections between the fragmentation, vacuolation and cephalosporin-c production in *Acremonium chrysogenum*

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Nowadays, one of the most widespread antibiotic from the 13-1actam family is the cephalosporin-C, produced by the filamentous mould *Acremonium chrysogenum*. Cultivating in batch cultures, cells exhibit various morphology. Besides the long vegetative hyphae, metabolically inactive arthrospores and wide, swollen hyphae fragments named yeastlike forms can also be found. The proportion of these cell types changes during cultivation. Morphological changes inside cell can also be realized as the vacuoles hardly observable at the beginning of fermentation virtually reserve the whole cell as time goes on, and later significantly widen them.

In this series of experiments we report data on the fragmentation and vacuolization of an industrial *A. chrysogenum* strain in minimal medium with glucose and sucrose in the presence and absence of methionine. We established that,

1) The strain is not an auxotroph for methionine, although in its presence significantly higher biomass production could be achieved.

2) During fermentation the proportion of yeastlike cells increased with time independently of the quality of medium. However, the quantity of changes was influenced by the carbon source used and by the presence of methionine.

3) Fragmentation of cells starts alter the late exponential phase, in contrast with the vacuolization, which seemed to be correlated rather with the depletion of the carbon source.

4) The consumption rate of methionine is negligible, therefore it is not used as a carbon or sulphate source. Its indirect effect is the acceleration of sugar consumption, which might explain the emerged biomass production as well,

5) In the case of a more effectively utilizable carbon source like glucose, the cephalosporin-C production is about twice as high as it is in the case of sucrose. In the presence of methionine, the specific antibiotic production is about four times higher compared to the methionine deficient medium independently of the carbon source.

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Chitinases in *Penicillium chrysogenum*

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Recently we found that *Penicillium chrysogenum* produces hydrolases (proteases and N-acetyl- β -D-hexosaminidase) during the aging of the cultures, therefore to know more about the produced enzymes especially hydrolases we investigated the production of a third type of hydrolase: chitinase. We compared the chitinase production using chitin carbon source and glucose carbon source in Vogel salt medium and complex medium. During the aging on glucose carbon source *P. chrysogenum* produces chitinase activity in a great extent. We determined the basic characteristics of the production and stability of the chitinase activity. Apparent molecular weights for chitinases were studied with non-reducing SDS-PAGE supplemented with glycol chitin as substrate.

Microsomal, cytosolic and extracellular N-acetyl- β -D-hexosaminidase and chitinase activities were compared in samples from the exponential and autolytic phase of growth in complex medium supplemented with glucose. The ratio of the increase in the activities between the two phases of growth was found to be similar. From the samples originated from the exponential phase zymogen chitinase activity was activated by protease treatment, this activation could not be obtained in the autolytic phase. From the autolytic phase extracellular chitinase activity specifically bound to the chitin were partially purified using ammonium sulphate precipitation and affinity chromatography. Four chitinase bands were found to bind specifically to the chitin column with 54, 41, 38 and 33 kD apparent molecular weights.

AGRICULTURAL AND FOOD MICROBIOLOGY

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Production of Shiitake (*Lentinus edodes*) mushroom and investigation of bioactive compounds

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Submerged culture of edible fungi have two main applications, as starter cultures (the spawn) for inoculating growth substrates and as food or flavour components in food products. In addition to its use as food, *Lentinus edodes* has been reported to have a number of beneficial health and medical effects (antiviral and antitumour activities).

The aim of this work was to find an economical method to produce Shiitake mycelium in submerged liquid culture (not by the traditional technology), and then to study whether the bioactive compound – eritadenine – and the flavour components (the main component lenthionine) were produced by *Lentinus* mycelium. All these compounds were identified in fruit-body by Japanese researchers earlier. *Lentinus edodes* mycelium was successfully produced in the fermentation experiments, in submerged culture (biomass productivity was 1.0–1.2%) and a considerable reduction could be achieved in the time required for biomass production as compared to the traditional technologies. On the basis of the GC-MS measurements it was proved that eritadenine, the bioactive compound was produced in the Shiitake mycelium. Based on NBS library data lenthionine, trithiolane and other aroma compounds were identified in the biomass and in the mushroom as well.

G. FONYÓ and K. SZENTPÉTERY

**A rapid detection of *Escherichia coli* from brewery samples
by a fluorescence method**

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In the food industry among indicator microbes the detection of coliform, and *Escherichia coli* bacteria is the most frequently used procedure. We compare the traditional method with the fluorescence method, already used in foreign laboratories. The rapid method is based on the indication of β -glucuronidase, produced by *E. coli*. While the traditional method needs at least 96 h at best, 24 h are enough to detect *E. coli* with the rapid technique. The test proved that the rapid method can be well adopted to brewery samples with special additional flora, and beside being quicker, sensitivity can occasionally be higher. This new microbiological method was found suitable to introduce it into brewery practice.

Z. BOZSÓ, P. G. OTT, M. KECSKÉS JR. and Z. KLEMENT

**Effect of compatible and incompatible bacterial
pretreatments on the necrosis inducing ability and HRP gene
activity of HRP mutants of *Pseudomonas syringae*
pv. *Syringae***

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The hypersensitivity and pathogenicity (*hrp*) genes of phytopathogenic pseudomonads are either involved in the development of disease symptoms in host-plant or in the induction of a rapid cell death of plant cells (hypersensitive reaction, HR) in non-

hosts. The pathogenicity of *hip* mutants are considerably decreased and these strains have lost the ability to cause HR.

In our earlier works we have shown that seven *hip* mutants of *Pseudomonas syringae* pv. *syringae* 61 (*P.s.* 61) were not able to induce visible HR on tobacco. However, if we temporally inhibited (with heat treatment or cycloheximide) the protein synthesis of the plant, one of the bacterial strains (the *hrpK* mutant) could cause HR. These treatments led to higher activity of several *hrp* genes than the control, as measured by β -glucuronidase (GUS) promoter probe reporter.

In recent experiments we have investigated if there are other treatments of leaves were infiltrated with *Pseudomonas syringae* pv. *tabaci* (*P. tabaci*, *Pseudomonas syringae* pv. *phaseolicola*) incompatible or *Pseudomonas syringae* pv. *pisi* (incompatible) pathogens. After that the leaves were challenged with *hrp* mutants of *P.s.* 61. In leaves pretreated with *P. tabaci* the *hrpK* mutant of *P.s.* 61 could induce HR similarly to tissue which were treated by protein synthesis inhibitors. The heat-killed *P. tabaci* did not cause this effect. The length of the period which was surfs, lent between pretreatment and challenge depended on the concentration of *P. tabaci*. After this pretreatment some *hrp* genes of *P.s.* 61 mutants, beside the *hrpK*, showed higher gene activity than the control. When tobacco leaves were pretreated with incompatible bacteria the *hrpK* mutant could also cause tissue necrosis. (in this case we prevented the incompatible pathogen from inducing HR itself). In addition, this pretreatment helped to increase *hip* gene activity. A feasible explanation for the behaviour of mutants, that the treatments (protein synthesis inhibition of plants or bacterial pretreatments) caused outflow of some materials (e.g. nutrients) from plant cells to intercellular space. it is also possible that these pretreatments may inhibit a HR independent resistance mechanism (early induced resistance, EIR), which would otherwise obstruct HR.

¹P. G. OTT, ²L. SZABÓ, ¹Z. BOZSÓ, ²E. BALÁZS, and ¹Z. KLEMENT

**Ultrastructure of the interaction between a HRP mutant
of *Pseudomonas syringae* pv. *phaseolicola* and tobacco leaf
cells showing locally induced resistance responses**

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The importance of studying the ultrastructure of bacterium-plant interactions has been refreshed from time to time by possibly new implications coming from other experimental areas, such as pathophysiology or signal transduction. It was shown earlier that other plant protective responses besides – and even counteractive to – hypersensitive reaction (HR) are induced. Such responses are the early induced resistance (EIR) and the late induced resistance (LIR). Both can be triggered by various types of bacteria but differ in requirements for developing time and light. Thus, EIR and LIR are inhibited by heat shock and absence of light, respectively. Using transmission electron microscopy, here we tried to associate some ultrastructural events occurring in and on leaf cells with EIR or

LIR. We inoculated into tobacco leaf panels a *hrp* mutant of the bean pathogen *Pseudomonas phaseolicola* that induces the EIR and later the LIR, but has lost the ability to induce the HR. In the control tissues localized cell wall appositions opposite to bacteria were observed inside the plant cell wall from 12 h post inoculation (hpi). Dark treatment was inhibitory to the development of these appositions, while heat shock caused only a minor delay. The treatments also affected bacterial morphology and multiplication, with the dark treatment bringing less changes and finally (at 48 hpi) being more permissive to bacterial growth, as compared to the control or heat shocked leaves where bacterial degradation was extensive at this time. Irrespective of time, the majority of events also depended on the microcolony size reached by the bacteria. Observations of bacterial entrapment onto plant cell walls were less conclusive for the treatments, but some general trends were discernible in time. It also appeared that both physical and physiological events contributed to the envelopment process. Since in general, specialized responses to bacteria were more affected in the dark than by heat shock, we think that the ultrastructural events examined are more likely linked to the LIR than to the EIR.

E. TÓTH

**Microbiological investigations into wound myiasis
of SHEEP caused by if *Wohlfahrtia magnifica*
(diptera:sarcophagidae)**

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Myiasis is responsible for serious agricultural losses all over the world through the devastation of the live-stock. From the parasites responsible for "myiasis sensu stricto" or wound flystrike in the temperate zone, diseases caused by *Cochliomya hominivorax* and *Lucilia cuprina* were studied in detail. Unfortunately we have only few information about the biology of myiasis caused by *Wohlfahrtia magnifica*. This parasite gives troubles to stock-breeders in our country as well, because according to the experiences up to the present *W. magnifica* is responsible for nearly 100% of the infections in Hungary. This fly is an obligate parasite of warm-blooded animals, it induces wound myiasis on different domestic animals (sheep, goose, cattle etc.). In Hungary a significant part of the sheep-stock is involved. In 1991 by examining 700 ewes the infection rate amounted 14%, in 1992 the ratio of affected animals has increased up to 40% in a flock.

As a result of the first bacteriological studies on *Wohlfahrtia* wound myiasis of sheep it became obvious that dramatic changes in the cutaneous microbial populations occur during or even preceding the fly infection. Although we do not know exactly what kind of visual and chemical stimuli are involved in recognition of the preferred areas for larviposition by females, we could prove that different chemicals (e.g. methane-thiol) produced by some dominant skin microorganisms (*Brevibacterium* sp.) attract the females of *W. magnifica*.

In 1995 we started detailed bacteriological studies on 8 different sample specimens obtained from the most susceptible regions of sheep to compare the aerobic and

facultatively anaerobic bacterial consortia of different individuals. (Among them there were males and females, healthy animals and myriatic regions containing larvae of different stages.) Strains were collected by the conventional plating and isolation methods using selected general purpose and differential media.

There were significant differences in the CFU numbers of bacteria among samples, however in all cases the absolute dominance of Gram-positive organisms could be proved. In ram preputium samples we could also show the massive presence of mycelial prokaryotes (e.g. nocardioform actinomycetes and even streptomycetes) and Gram-positive cocci. In females there were sample specific differences but identities, too: Gram-positive cocci and different coryneform bacteria dominantly occurred. The ratio of obligately aerobic versus facultatively anaerobic microorganisms varies in the different samples.

A. HALBRITTER and G. KOVÁCS

**Phenotypic characterization of bacterial communities
inhabiting the rhizoplane of *Typha angustifolia* living in
floating wetland**

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In Hungary there had been an enormous abundance of different wetland ecosystems. Many of them were floating ones: fens or bogs on floating peat mat. By the 60's of our century most of them have been destroyed mainly due to human activities (first of all agriculture), neglecting their important role in self-purification processes of natural waters. Generally, wetlands are important components of the biosphere and play a significant role in the global cycling and geochemical balance of water, carbon, nitrogen, and sulphur.

Until now there has been some research on the flora and fauna of floating wetlands, but we have extremely scanty knowledge on their microbial communities, however the biogeochemical and self-purification processes are based mainly on microbial metabolism. Also the wetland macrophytes strongly depend on their microbial environment under the extreme anoxic conditions. On the other hand, oxygen-releasing aerenchymatous plants may affect the composition of the microbial community in the waterlogged peat pseudosoil. Hence, we focused our investigations on the rhizoplane bacteria (as the closest microbial partners of the dominant plant species).

The sampling site was in a floating fen dominated by narrow-leaved cattail (*Typha angustifolia*) of the Danube-arm of Soroksár. Following a short light and scanning electronmicroscopical study on *Typha* root aerenchyma and surface (rhizoplane), more than 1000 rhizoplane bacterial strains were isolated. Out of them 210 were characterized in detail by their morphological features, biochemical and ecophysiological properties. Based on these phenotypical data computer aided numerical analysis was performed. Similarity calculations were made by the simple matching coefficient, and clusters were

generated by using the UPGMA-method. Typical strains of each phenon were also characterized and parallelly determined by the BIOLOG metabolic fingerprint assay.

Most of the strains investigated were Gram-positive, and 60% of them were strictly aerobic. In addition, approximately 80% of these strains exhibited an obligatory oxidative metabolism. As the result of our investigation work the predominant phenons belong to the following genera: *Acinetobacter*, *Enterobacter*, *Flexibacter*, *Bacillus*, *Micrococcus*, *Staphylococcus* together with different coryneform types. Hence it can be concluded that oxygen itself might be an important environmental factor in selecting the dominant bacterial populations. A significant portion of the strains showed denitrifying activity or growth on N-free media. The mixed rhizoplane community exerted in vitro sulphide oxidation in gradient cultures. This capability may protect plant roots against toxic effects of H₂S.

J. MÁTÉ and I. LENTI

Tinders from the Bátorliget ancient bog

Agricultural College Faculty of Gödöllő University, Nyíregyháza

The living world of Bátorliget is the richest among the survived or lately perished bogs in Hungary. This wide biodiversing might have remained from beech age, the woody – boggy – bare age of the Great plain, but certainly its birch – bogs are originated from the birch-pine age. Some varieties relate to the glacial origin (Soó, 1953). According to our opinion, this interesting, multi-coloured flora was followed as devoted attendant by special creatures, the fungus, which were forgotten in the respect of the research work. The sharpest representative with large body are the traders in this biotop. Ubrizsy (1953) partly elaborated the "Quasimodos" of the Bátorliget ancient bog, but his work has to be continued. During the fungus monitoring of the spring aspects in 1995–96, we tried to lay down – after more than forty years – the results of Professor Ubrizsy. Our purpose was to complete them and to sign the absences of those species, which were written by him. During our examination we verified that there are fifty-four tinder varieties from the written ones in the living community. We could not have verified the presentation of twenty-three species, but we could have found some new ones. These are the followings: *Leptoporus* genus, 3 independent species; *Trametes* genus, 3 independent species; *Oxyporus* genus, 1 independent species; *Fomes* genus, 3 independent species; *Phellinus* genus, 3 independent species; *Inonotus* genus, 2 independent species.

J. MÁTÉ and I. LENTI

Morel fungus of the Bátorliget ancient bog*Agricultural College Faculty of Gödöllő University, Nyíregyháza*

The surviving of the ancient living world has been resulted by the local microclimatic relations in the Bátorliget ancient bog. The cold underground water, moving near the surface, moistens and cools the soil, so the air-layer near the soil remains fresh (Soó, 1953).

The evaporation of the bog-water results a misty air, the near forest-wreath prevents carrying toward of the developed veil of mist – even on the hot summer days. Our fungus species propagate excellently in this special, almost unique climatic and edafic surrounding. Our discovering work in the spring of 1996 was directed for determination of the fungus-joinings of the spring aspects. We verified that three fungus genera represent the morel fungus in the Bátorliget ancient bog. These species are growing from the beginning of April to the end of May, and considerable number of them can be found in the forest and its surrounding. The bonited species occur randomly, they do not have a well framed growing place. They can be found in the dry fallen leaves and parched grass of the oaks, willows, poplars and ashes, and on the grassy clearings. The discovered species were: *Morchella esculenta* (L.) PERS., *Morchella conica* PERS., *Mitrophora semilibera* (SOW.) SCHROET., and *Verpa bohemica* (KROMBH.) SCHROET.

J. MÁTÉ and I. LENTI

Tremella, Exidia and Auricularia* fungus species from the Bátorliget ancient bogAgricultural College Faculty of Gödöllő University, Nyíregyháza*

T. Simon (1956) did not make a mistake, when he formulated that the results of the phytological and zoological investigations proved that Bátorliget was a very interesting area – protecting the Great plain – and this statement is also true in 1996.

The botanical and animal world of the Bátorliget ancient bog is mostly well-known and perfectly discovered. But this activity must not be finished by the floristical and faunistical investigations. We are obliged to professor G. Ubrizsy, who knew and exerted a considerable activity that the representatives of our natural ornaments – which have a big scientific value – could enrich the store of our knowledge, because the fungus are part of the biotop. We aimed to continue his work, published in 1953, in which he described one hundred forty-four species of seventy-four fungus genera. The scientists of the Agricultural Department of the Agricultural College have formulated the mycological investigation of the Ancient Bog. They have started the discovering with the help of Hungarian and foreign specialists in the winter of 1995. The scientific results of the

winter aspect: the species of the *Tremella* genus: *Tremella mesenterica* RETZ. and *Tremella faginea* BRITZ.; the species of the *Exidia* genus: *Exidia glandulosa* (BULL.) FR.; from the *Auricularia* genus: *Auricularia mesenterica* (DICKS.) FR.

¹G. SZAKÁCS, ¹K. URBÁNSZKI, and ²J. FEKETE

Isolation and characterization of xenobiotic degrading bacteria

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Bacteria capable of degrading hazardous chemicals were isolated from polluted soil and sewage sludge originated from a chemical company. A two step laboratory enrichment procedure was applied in shake flask before isolation. The quantity of chemicals in enrichment medium was 300 ppm. Biodegradation efficiency was followed by high performance liquid chromatography (HPLC) Reverse phase HPLC coupled with UV-diode array detector and personal computer (PC) was used for monitoring. Most promising mixed and pure cultures have been freeze-dried. The following target compounds were used for biodegradation studies: 4-chlorophenol, 2,4-dichlorophenol, 2,6-dichlorophenol, 2,4,6-trichlorophenol, acetochlor, atrazin and AD-67. Identification of bacteria capable of partial or total degradation of the above compounds showed that dominant isolates belong to the following species: *Agrobacterium radiobacter*, *Bacillus coagulans*, *Bacillus mycoides*, *Bacillus sphaericus*, *Pseudomonas aeruginosa* and *Pseudomonas putida*. During the continuation of this work, these isolates may be used in bioremediation studies.

CS. ROMSICS

Examination of xenobiotics-degrading microbes by classical microbiological and molecular biological methods

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Environmental pollution is one of the most threatening problems at the end of the 20th century. The accumulation of certain aromatic compounds (benzene, toluene and xylene the so-called BTX compounds), some of the different halogenated pesticide etc. combinations, produced by the "modern" chemical industry (heptachlor, lindan, mirex), and other aliphatic and aromatic hydrocarbons from "simple oil spills" in our soils and water is accounted as the biggest danger. These extremely hazardous compounds are eliminated from the biosphere by different, usually slow physicochemical pathways,

which in almost all occasions can be enhanced and accelerated by microbiological activities.

The role of microorganisms is very important in the biodegradation of pollutants. It is well known that the adequate xenobiotics-degrading microbes rapidly multiply in polluted areas in contrast to nonpolluted ones. There are different factors that certainly play a role in the rapid adaptation and multiplication of the microbiota, for example selection processes, mutation, and perhaps, the horizontal gene transfer among the microbes, too (e.g. conjugative plasmids carrying the xenobiotic-degradation capabilities are responsible for a part of the horizontal gene transfer).

Forty-eight bacteria were isolated from diesel oil polluted soils supposedly having high biodegradation capabilities of hydrocarbons (due to a long term, continuous spill). Their taxonomic position was characterized at generic level by using classical diagnostic methods. The hydrocarbon degradation pattern of our strains was examined in test, where different hydrocarbons were applied as sole source of carbon and energy. The presence of extrachromosomal genetic elements (plasmids) was investigated in case of strains degrading wide range of hydrocarbons.

The dominant portion of our strains belongs to the genus *Pseudomonas*, while an other important group consists of members of the different coryneform genera. On the basis of plasmid exclusion experiments, we suppose that the biodegradative capabilities are encoded on chromosomal genetic material.

I. TÓTH

Effect of forest fire on microbial communities of soil

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Forest fire is considered recently as an important ecological factor, not only as a destructive force. Some ecosystems can adapt to the fire and what is even more important, fire is necessary for their survival and health. It is indisputable that investigation of the development of new microbiota or even tracing of regeneration of microbial associations in a burned area, is a useful and extremely interesting research field. The first profound analysis of a burned forest was published on the catastrophe stricken territories affected by the volcanic eruption of Mount St. Helen. It became evident, that the fire affects the environment deeper than its visible effect, the total or partial destruction of vegetation, the fire really alters the soil properties, the nutrient cycle, activity and number of microbes etc.

In the hot summer of 1993 a planted *Pinus nigra* forest near Pilisvörösvár had been destroyed by fire. In some sites fire has consumed the forest totally, even the only remained trunks, together with the soil were carbonized, on other sites total or partial tree crown bums were detected, whereas in some forest parts only the litter layer has been destroyed and trees survived alive. Headed by Prof. Zoltán Járó (Forestry Research Institute) a study group started to measure the effects of fire on soil properties and vegetation, to which members of the Department of Microbiology have also joined. Three

plots affected by fire in different degree were selected for study and an unburned site as control area.

On 13th and 16th October of 1994 in all plots, soil samples were collected from different depths at characteristic sampling places. The goal of our study was to determine the total CFU counts of bacteria and specially the number of nitrifying denitrifying and free living nitrogen fixing bacteria, and also to identify, if possible the dominant N_2 fixing organisms. Total CFU counts were detected by using traditional plating on meat-peptone and starch-casein agars. There were no significant differences between burned and control samples. The counts of nitrifying, denitrifying and nitrogen fixing microbes were determined by the MPN (Most Probable Number) method. It can be concluded that in the burned areas the number of ammonia oxidizers significantly increased, regarding the numbers of denitrifying bacteria we noticed only a small increase, and the counts of nitrogen fixing microbes decreased with at least one order of magnitude. Isolations were made on Ashby nitrogen-free agar, and the strains were examined for their morphological features and biochemical capabilities moreover they were characterized by the BIOLOG metabolic fingerprint method too. The dominant portion of the strains was identified as members of the genus *Pseudomonas* and only a small fraction of our strains belonged to the genera *Agrobacterium*, *Rhizobium*, *Xanthomonas*, *Cytophaga* and *Bacillus*. The results of acetylene reduction assay however showed that a very low proportion of the strains were capable of actively fixing nitrogen.

A. MOHAMED SALEM and K. MÁRIALIGETI

Studies on the autotrophic bacterial communities of Danube river

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Investigations on the autotrophic bacterial communities of the river Danube seem to be totally neglected. This fact is most possibly connected with the experimental difficulties, the time and labour consuming isolation, characterization and identification work. However one has to realize that these bacteria are usually in key position in the biogeochemical cycles of different elements, due to their special biochemical activities. The aerobic H_2 oxidizing bacteria ("Knallgas bacteria") e.g. effectively control the interspecies H transfer in biofilms, thus influence biofilm sloughing. From the point of view of drinking water quality ammonia and nitrite oxidizing bacteria (i.e. nitrifying consortia) play an utmost importance by increasing the mobility of inorganic N compounds, etc. Our investigations were focused on the isolation and identification of the Danube autotrophic bacteria.

Danube water and sediment samples were collected several times, partly plated directly onto different mineral media, and partly used for inoculation of enrichment culture fluids. Two weeks to three month old enrichment cultures were used directly for plating, and at the same time isolations were made by the method of serial dilution too. As a result of different isolations more than 300 agar cultures and about 200 broth cultures

were obtained. All of the isolates were purified, and subcultured onto different mineral and complex, broth and agar media containing diverse C and N sources. Thus our strains were grouped, concerning their cultural characteristics into four metabolic types. The first ones were which grow well in mineral media, but can be cultivated in the presence of carbohydrates (e.g. glucose) also. These are without exception facultatively H_2 autotrophic bacteria, dominated by the species *Hydrogenophaga palleronii*, *Xanthobacter agilis* (carotenoids with special absorption spectra help identification), and *Acidovorax* sp. The second group comprised of oligocarbophilic-methylotrophic strains, utilizing organic acids (e.g. pyruvate) as C source, and naturally CH_3OH as sole source of C and energy. The predominant biofilm phenon was composed of representatives of *Hyphomicrobium zavarzinii*. (The identification has been corroborated by 16S rDNA sequencing, too.) The third group unified the NH_3 oxidizers, dominated by *Nitrosomonas* and *Nitrosococcus* species. The last group contained NO_2^- oxidizing microbes, with predominant presence of *Nitrobacter* sp. In case of nitrifying bacteria the genetic characterization (based on 16S rDNA sequencing) has been started.

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Interaction between *Rhizosphere* microorganisms and red clover at metal stresses

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Some harmful effects of various environmental stress factors (such as salinity, pH, metal tolerance etc.) on the beneficial symbiotic *Rhizobium* bacteria in vitro have already been published [1, 2]. This paper is concentrating on the in vivo interactions between the rhizosphere microorganisms and red clover (*Trifolium pratense* L.) stressed by heavy metals under greenhouse conditions.

Calcareous chernozem soft originated from a long-term field experiment of Nagyhorcsök, Hungary. The soil was artificially contaminated by 0, 90, 270 and 810 kg ha^{-1} Zn, Ni and Cd salts (sulphates) five years ago in 1991. Fresh and dry weight of red clover, the rate of endomycorrhizal (AMF) infection, *Rhizobium* nodulation, N_2 -fixation (acetylene-reduction activity-ARA), MPN counts of associative *Azospirillum* bacteria, number of the fluorescens *Pseudomonas* and inorganic phosphate-solubilizing bacteria were examined after 12 weeks of cultivation.

It was established that in the gamma irradiated, sterilized soil without rhizosphere microorganisms the fresh and dry weight of clover was less by 70% comparing to the original soil-plant system. Zn concentrations below the European Permissible Limit had no reduction on the root nodulation and dry weight, although there was a negative influence found on the phosphorous content of samples. Cd was especially harmful for the microsymbionts and phytotoxic for the clover. Significant reduction of associative azospirilla was realized with the increasing Ni applications.

After three months of cultivation the number of total bacteria, fluorescent pseudomonads and phosphate solubilizing bacteria increased to the same level as in the unsterilized treatments, but the fresh weight of plants without a starter positive effect remained less. Although endomycorrhizal colonization was only slightly influenced by the various metal rates, the number of AMF arbuscules were found to be decreasing as a function of harmful metal rates. This parameter proved to be a good indicator of metal stresses among the various environmental conditions.

[1] Bíró, B. et al.: In: *Azospirillum* 6, NATO ASI Series, Vol G37. Springer Verlag, Jena, 1995. pp. 495–500.

[2] Bayoumi, H.E.A.F. et al.: *Acta Biol Hung* 415, 17 (1995).

¹I. FÁBRI, ²CS. MOHÁCSI-FARKAS, and ³J. BECZNER

The assurance of food safety and stability by means of HACCP system

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The “Guidelines of the Application of HACCP system” to ensure the safety of food was edited by FAO/WHO Codex Alimentarius Commission in 1993 and its adoption in the food industry is mandatory in the European Union and in the USA. The essential basic implementation of HACCP is the application of the “General Principles of Food Hygiene” worked out also by the FAO/WHO Codex Alimentarius Commission. It is obvious that the pathogenic and spoilage microorganisms are the most important hazardous factors threat the food safety and stability, its suitability for consumption. To prevent the harmful life-functions of the microbes require a thorough research work in the field of microbial ecology, biotechnology, gene-technique etc. moreover the development of rapid methods etc. The basis of these microbiological work has been established in Hungary since decades, the next task is a continuous development

K. FORGÁCS, J. KERTÉSZ, and B. LENKEY

Partial comparative investigation of fluconazole sensitive and resistant *Candida albicans* strains

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Candida albicans is an opportunistic pathogenic yeast. Because of its potential ability for infection its importance has increased in the last ten years. The frequency of life threatening fungal infections is rising world-wide. This trend is caused by the increasing use of immunosuppressive and cytotoxic drugs, and more powerful antibiotic agents; the global HIV/AIDS pandemic has also had a major impact on the incidence of serious fungal infections. Numerous antifungal agents have been developed, but in most of the cases quickly appeared less sensitive, resistant strains.

It seemed advisable to compare fluconazole sensitive and resistant strains with the less genetic differences possible, first of all in respect of morphological features and virulence factors. So we partially compared a fluconazole sensitive strain (MIC 5 µg/ml) and a fluconazole resistant strain (MIC >80 µg/ml) which had been prepared from the sensitive one. We investigated the growth of the two strains on two complex media containing different components, and we have found differences between the speed of growth. Morphologically we examined cell-size, adherence to human buccal epithelial cells, and germ tube formation. The examination of germ tube formation was performed on human and on sheep serum. On the basis of our measurements the ability of fluconazole sensitive strain to form germ tubes considerably differs from that of fluconazole resistant strain.

Among virulence factors we examined extracellular aspartic proteinase. Proteinase activity per number of cells of the fluconazole resistant strain is nine-times higher than that of the fluconazole sensitive strain. Based on this fact we determined MICs to fluconazole and extracellular aspartic proteinase activities of numerous *C. albicans* strains (ATCC strains, and clinical isolates). Forming three groups on the base of MIC values (sensitive, moderately resistant, and resistant) we can say, that sensitive strains to the antifungal agent have the smallest average aspartic proteinase activity, fluconazole resistant strains have the greatest, and moderately resistant strains have middle proteinase activity. Besides we examined the inhibition of extracellular aspartic proteinase enzymes of the fluconazole sensitive and fluconazole resistant strain with ammonium-ions, and proteinase activity of two strains at different fluconazole concentrations, and we have found significant differences.

¹A. HEGEDŰS, ²S. FARKAS, ³GY. VÁRADY, and ⁴M. KECSKÉS

**Effect of *Pseudomonas* and *Fusarium* interactions
on the growth of wheat**

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Siderophore *Pseudomonas* strains (PGPR) cultivated in King B liquid medium. With suspension of ten strains' mixture were treated the seeds of wheat (cultivar "Tavaszi") for 5 min. Pathogenic *Fusarium* strains were grown in Martin liquid medium. Fifteen wheat seeds were sown in each plastic pots, containing sterile perlite medium a one cm deep layer of which was infected by *Fusarium* suspensions. In the second treatment the infected perlite layers were covered with one cm sterile perlite layer. Sowing was carried out on the non infected layer. Emergence and the state of plants were measured per day. The quantity of *Pseudomonas* cells on the surface of wheat seeds was determined by dilution method before sowing then daily. The seedlings were destroyed at percentages: control 5, treated with *Pseudomonas* without *Fusarium* 0.8, infected with *F. graminearum* without *Pseudomonas* 88, infected with *F. oxysporum* without *Pseudomonas* 100, infected with *F. graminearum* treated with *Pseudomonas* 45, infected with *F. oxysporum* treated with *Pseudomonas* 22.

¹L. KARAFFA, ²J. KOZMA, and ¹A. SZENTIRMAI

**The role of the enzyme isocitrate lyase in the respiration
of *Acremonium chrysogenum***

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The main producer of the antibiotic cephalosporin-C is the filamentous mould *Acremonium chrysogenum* which, under industrial circumstances, is cultivated on complete medium in the presence of different carbon sources like sugars and fatty acids. The goal of this study was to elucidate, when and how the soybean oil, essential in number of fermentation processes is metabolized during fermentation. The following results were obtained:

1) On complete medium with soybean oil, higher biomass production can be achieved even in the early stages of cultivation.

2) In the presence of soybean oil, thenoyltrifluoroacetone (TTFA, specific inhibitor of succinic dehydrogenase (complex II)) significantly (over 50%) decreases the respiration activity of cells in the first 24 h of cultivation. Later, this inhibitory effect starts to decrease until the 40th h, when no inhibition can be observed. In the absence of soybean oil, there is no significant reduction in respiration in the first 24 h either.

3) Mycelia washed into a minimal medium with succinic acid as a carbon source exhibited similar (75%) sensitivity against TTFA like cells washed into an only soybean-containing minimal medium. In contrast, when cells were transplanted into an only glucose-containing minimal medium, the inhibitory effect remained small (25%).

4) In the first 36 h of fermentation, a gradually decreasing isocitrate lyase activity could be assayed when the complete medium possessed soybean oil, while no activity could be measured when lacked it.

To sum it up, it was established, that the soybean oil has a significance in the first stage of fermentation. Its degradation presumably happens in the glyoxalate cycle. The succinic acid produced by this process seems to be the most important substrate of terminal oxidation at this early stage.

Z. KISS

Inorganic phosphate-mobilizing activity of strains of different *Bacillus* species

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To study of inorganic phosphate-solubilizing microbes (IPSM) is very important because: of lack of soluble inorganic phosphate in soils. Occurrence of IPSM in terrestrial ecosystems, their PO_4^{3-} -solubilizing capacity and ecophysiological characters are necessary from practical (plant inoculation) as well as oriented basic research points of view. More than 130 bacterial strains were isolated from 21 types of soils of seven main soil types of different regions in Hungary; 13 strains were chosen for further investigations. These are representing the species: *Bacillus macerans*, *B. azotoformans*, *B. laevolacticus*, *B. megaterium*, *B. insolitus*, *B. brevis* and *B. firmus*. They will be selected on the bases of their ecophysiological characters. Their inorganic phosphate-mobilizing capacity was tested on solid (Pikovszkaya and Razumovszkaya media) and. liquid media. The investigated strains were selected on their ecological characters and were registered according to their increasing order of their solubilizing activity. There was no significant difference concerning the pH changes of the nutrient solutions, but the pH values decreased in every cases. Further selections are being in process after this in vitro investigation to test the phosphate-solubilizing ability of *Bacillus* strains for using as potential plant inocula among various environmental conditions.

¹Z. KLEMENT, ¹Z. BOZSÓ, ¹P. G. OTT, and ²R. KLAUS

**The mechanism of symptomless reaction of plants induced
by pathogenic pseudomonads**

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Leaf spot inducing pseudomonads usually cause a hypersensitive reaction (HR) in non-host plants. In certain cases, however, the necrotic response does not occur. The mechanism of this symptomless "immune" response was not known before. We have shown, that a single bacterial inoculation induces two local host responses at an early stage of pathogenesis. One of these is the HR and the other is the early induced resistance (EIR) of plants. It was found that the symptomless response is a consequence of the development of the EIR prior to the induction of HR. This situation exists either a) when the EIR develops very quickly (e.g. at high temperatures) or b) when the HR induction time of pathogens is delayed (e.g. in some pathovars or mutants). We have results supporting both cases: a) the EIR developed in tobacco leaves at 20 °C by 3–6 h, but at 30 °C by 1–2 h. Therefore, *Pseudomonas syringae* pv. *phaseolicola*, the HR induction time of which is rather long (2.5–3 h) at both temperatures, causes HR at 20 °C but not at 30 °C; b) the induction time of some Tn5-mutants of pv. *phaseolicola* is delayed. During this longer induction time of HR (3–6 h) the EIR can develop also at normal temperature (20 °C). Therefore, the hypersensitive necrosis does not appear. These findings were verified in experiments in which the length of the HR induction time was extended by chloramphenicol treatment of wild strains of pv. *phaseolicola*. When the development of EIR was inhibited in tobacco leaves by inhibition of plant protein synthesis (cycloheximide or heat shock at 50 °C for 13 s), the HR appeared.

G. KOVÁCS

**Taxonomic investigations into the rhizoplane bacterial
communities of *Typha angustifolia* by 16S rDNA sequence
analysis**

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The goal of our experiments was to get an insight into the taxonomic composition of the root-surface microbiota of *Typha angustifolia* (narrow-leaved cattail), which is in floating bog plant associations a characteristic plant species, usually the dominant macrophyte. Samplings were made in three different seasons, in Summer, Spring and Autumn. Root tip regions were collected, serially washed in sterile water, smashed, and suspended, thereafter plated on the surface of starch-casein and peat extract-yeast extract agar media, incubated. The developed colonies were isolated on a random manner. From among more than 600 isolates 210 strains were chosen for detailed investigations. Based

on the results of morphological characterization, and biochemical-physiological testing, cluster analysis was performed. Characteristic representatives of all fenons (all together 75 strains) were selected for 16S rDNA sequencing. The 16S rDNA regions of DNA extracts made from 24 h old cultures were amplified by PCR technique, using specific primers. Following a purification the PCR product has been sequenced by the Taq Dye-Deoxi Cycle Sequencing Kit. In this latter case the manufacturers prescriptions were followed. The results were evaluated by an Applied Biosystems 373A automatic sequencer. The sequences were prealignmented by using the ARB software, and identification was revealed by the comparison of the 16S rRNA sequence of our strains investigated with authentic sequences in 16S rRNA data bases. The overdominant proportion of *T. angustifolia* rhizoplane bacterial communities was composed of *Bacillus pumilus*, while other Gram-positives, like *Microbacterium lacticum*, *Dactylosporangium* sp., *Staphylococcus* sp., were in subordinate position. Among Gram-negatives different *Acinetobacter* spp. dominate, like *Acinetobacter lwoffii*, *Acinetobacter radioresistens*, while other species, such as *Erwinia herbicola* or *Agrobacterium vitis* type bacteria are present in a low ratio only. It is obvious however, that on contrary to average plant rhizoplane, where Gram-negative bacteria seem to dominate, in case of the wetland plant, *T. angustifolia* the rhizoplane communities can be characterized by Gram-positive organisms. No or low degree of seasonal variation could be detected. This predominant presence of *B. pumilus* possibly indicates a closer interaction between this prokaryote and its plant host. It has to be emphasized, that a cluster-analysis based on classical test methods seems to be adequate for a genus level identification of dominant populatory bacteria.

A. MARTIN

**Comparative investigation of thermophilic *Bacillus* strains
using classical methods and a new rapid identification test
system (Biolog)**

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Hungary is well known for the wealth of thermal waters. If possible, Budapest is in even more favourable situation due to its particular geological location. In the last several years not only the hygienic microbiological control of inflow well water in spas, but also knowledge on the autochthonous thermophilic microbial communities of hot waters has emerged in the centre of scientific interest.

The four hottest thermal wells of Budapest (water temperature around 70 °C, Széchenyi spa, wells No. I. and II, Magda well, Paskál well) were selected for investigation. The nutrient broth enrichment method by using different N and C sources followed by plating proved to be suitable for isolation (direct plating and the membrane filter technique had to be rejected). We could rank all of our 150 thermophilic isolates into the genus *Bacillus* based on the results of their cultural-morphological investigations and primary biochemical tests. These strains and seven thermophilic reference *Bacillus*

cultures (kindly provided by Deutsche Sammlung von Mikroorganismen) were submitted to a detailed biochemical study. Overall similarities of these strains based on 134 coded characters were determined by the S_{SM} coefficient and clustering achieved using the UPGMA algorithm. According to the results of our computer aided numerical taxonomic analyses, numerous metabolic types and varieties of a few *Bacillus* spp. constitute the major fraction of the indigenous thermophilic microbial communities in subterranean thermal waters of Budapest.

The Biolog 96-well microtitre plate metabolic fingerprint assay offers a standardized, rapid method for determining the bacterial oxidation of 95 different carbon sources simultaneously. In contrast to other rapid test systems based on colour changes of pH indicators, the Biolog technique relies on the irreversible reduction of a tetrazolium redox-dye to the purple formazan as an indicator for organic substrate oxidations. The reproducibility is assured by means of definite inoculum preparation methods. To prove, whether results from the Biolog system can be used for rapid and reliable grouping of bacteria not included in the system's data bases, representative strains of our isolates were evaluated for their carbon source oxidizing abilities on "Gram-Positive-Microplates". Comparison of the results with the conventional sole-carbon-source utilization tests showed considerable differences. The dissimilar results obtained with the different test methods are obviously due to their different mode of operation. However, the Biolog system is a useful tool for differentiation and grouping of large numbers of isolates, even if the use of Biolog data base can lead to incorrect identification of strains.

¹Z. NAÁR and ²M. KECSKÉS

Colonization of different soft types by *Trichoderma* fungi

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The colonization of soil is a key factor in efficiency of *Trichoderma* fungi applied in biological plant protection against soilborne pathogenic fungi. We studied the effect of physico-chemical parameters of different soil types, soil micro flora, competitive saprophytic ability and origin of isolates on colonization of soils by trichodermas.

Using conidial suspension for soil inoculation, trichodermas achieved significantly different colonization in different soil types. Chernozem soils were not colonized, whereas sandy soils were always grown over by the applied strains. In brown forest, salt affected, and meadow soils medium colonization values were observed. The type of soils had a stronger effect on colonization than the competitive saprophytic ability of strains. Humus ($r=-0.56$), $\text{NO}_3 + \text{NO}_2$ ($r=-0.48$) P_2O_5 -content ($r=-0.58$), and K_A ($r=-0.40$), showed significant correlation with colonization value. These factors may not directly affect the colonization of soil rather through the activity of microbial communities. Using inhibitors (antibiotics and fungicides), it was revealed that mainly josamycin-sensitive bacteria (mainly Gram-positive rods and cocci) and metalaxil-sensitive fungi (*Phycomycetes*, *Oomycetes*) are responsible for weak colonization of soils by

trichoderma. When soil samples were cross-inoculated with *T. virens* isolates, soils inoculated with own isolate (originated from the given soil sample) were colonized more effectively.

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**Bank-wall filtered well water quality as a function
of Danube rolling gravel bed biofilm bacterial species
composition**

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Although during the last 50 years the Hungarian reach of Danube has been intensively studied from different biological aspects, so far there are no or very few data regarding the autochthonous bacterial communities of the river. Health-related microbes seem to be the only exception, which are of course usually not indigenous, however the former ones play an important role in the self purification processes of the water. Moreover the natural microbial assemblages attached to the surface of the Danube bed rolling gravel particles, have a key function in the determination of the water quality in bank-wall filtered wells. The objective of our study was to recognize the impact of these autochthonous bacterial communities on the drinking water quality in Budapest, as drinking water in the capital is in more than 80% filtered through this biofilm.

Danube water, gravel and raw well water samples were taken at monthly intervals for over a year at Kisoroszi well No. 1. Samples were immediately transported to laboratory. More than 30 different routine water physico-chemical and biological parameters were detected to characterize the Danube and well water samples, moreover from well water, and gravel biofilm samples isolations were also made. Each month 50 well water and 50 biofilm strains were subjected to detailed morphological and biochemical characterization; cluster analyses were made, and representatives of the dominant fenons were identified at species level by using the Biolog metabolic fingerprint assay.

Based on our results we can state, that oligotrophic Gram-negative bacteria, like *Pseudomonas* and *Xanthomonas* spp. characterize well-water samples throughout a year. On the contrary biofilms were dominated by Gram-positive coryneform bacteria, first of all by *Rhodococcus*, and *Cellulomonas* spp. Changes in the population density of members of the latter genus are in good accordance with changes in the Danube water diatom counts. Members of the families *Enterobacteriaceae*, *Pseudomonadaceae*, *Vibrionaceae*, and the genus *Acinetobacter* were the characteristic Gram-negative biofilm bacteria. Seasonal changes were typical only in the biofilm bacterial consortia (pseudomonads in the cold winter waters were succeeded by aeromonads in the summer period). The dominant raw well-water bacterial populations usually differ from the

dominant biofilm populations, however in case of floods a "Danube effect" can be detected: i.e. characteristic biofilm bacteria (like *Rhodococcus* sp.) can temporarily also be detected in well water.

I. MOKHTAR and K. MÁRIALIGETI

Shift in the composition of sheep vulval skin anaerobic microbiota due to myiasis

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Wohlfahrtia magnifica (Shiener) is one of the main etiologic agents of cutaneous myiasis of sheep in Hungary. About 35–50% of sheep in a flock can be infested by the larvae of this obligatory parasite. Mortality rates among lambs are extremely high, often exceeding 15%. Unfortunately our present possibilities to control the spreading of this disease are limited. The use of adequate insecticides is not only expensive, but it is highly labour consuming. The analysis of the aerobic and facultatively anaerobic bacterial communities of myiatic lesions and the parasite's intestines strongly emphasized the role of bacterial population changes in the etiology of this disease. On the other side, from previous results we know exactly, that skin injuries deeply influence O₂ levels in different skin layers, and concomitantly the composition of the strictly anaerobic microbiota. The aim of our work was to study this possible shift in the anaerobic bacterial communities and its inferred effect on fly attractance, due to supposed metabolic alterations.

Samples were collected from healthy skin and myiatic wounds, in breech region of Romney breed ewes. Two methods were used for sampling: the "cotton-swab rubbing" and the "glass cylinder procedure". Samples were immediately transferred into anaerobic jars and any further operations (like plating, incubation, isolation, biochemical test, morphological examinations, etc.) were performed in an anaerobic glove system.

About 45 strains isolated from healthy skin and 60 strains from myiatic lesions were subjected to further detailed investigations concerning their key differential diagnostic characters. Our results suggest that a considerable shift exists in the species composition of anaerobic bacterial consortia of healthy skin as compared to myiatic skin. In healthy skin samples Gram-positive anaerobic cocci dominate, whereas in myiatic wounds extremely mixed bacterial consortia can be found, where different *Clostridium* species are characteristic. What concerns the composition of volatile fermentation end products, the qualitative composition seems not to be changed, however the molar ratios of the components extremely differ.

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**A preliminary attempt to model growth of bacteria
on pre-cut vegetables using conductimetric measurements**

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In relation to our studies on the effects of low-dose irradiation on microbiological safety and stability of pre-cut vegetables, changes of populations of aerobic bacteria on untreated and gamma-irradiated (1 kGy) pre-cut, prepackaged green bell pepper and carrot cubes were followed during storage at various temperatures both by plate counting and conductance measurements with a RABIT instrument. Comparison of aerobic viable cell counts and conductance curves at 30 °C incubation temperature showed that, under defined experimental conditions and the given range of storage temperatures, the log viable cell counts of the pre-cut vegetables (lg CFU/g) could be correlated well ($R^2 > 0.8$) for both the untreated and the low-dose irradiated samples with a combined function of the time to detection (TTD) of the first change in conductance of the RABIT medium, and the logarithm of the maximum rate of conductance change (lg MRC). Untreated and irradiated samples from the same batch of raw material could be characterized by the same equation ($\lg \text{CFU/g} = a - b \cdot \text{TTD} + c \cdot \lg \text{MRC}$). However, moistened (aqueous dip) vegetable pieces showed faster microbial growth than dry (non-dipped) pieces of the same vegetable, resulting in changes of the constants of this function. Further studies are started to check reproducibility of the mathematical model, the validity of the general function for other pre-cut, chilled vegetables, and the possibility of using a similar experimental approach for establishment of mathematical functions for eventual shelf-life prediction on the basis of conductimetric data on microbial populations.

B. C. O'HEIX, I. GRIGORSZKY, and P. OUDIN

**Algae growing on tree trunks as bioindicators of urban
air quality**

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The abundance of epiphytic green algae has been observed to correlation strongly with air quality in several studies. In contrast to lichens, the abundance of green algae is difficult to measure accurately by visual observation. The proposed method has been developed in order to estimate the chlorophyll content in algae growing on the bark of pine. Calibration data for the method was collected at polluted areas by spectrophotometry analysis of chlorophyll density. The aim of the study was to develop a non-destructive, reliable and replaceable method for the rapid measurement of the abundance of algae growth on tree trunks.

The epiphytic green algae have been noticed to increase in polluted areas. The algae grow first on tree trunk, but can later spread to the needles of conifers (Ahti and Vitikainen, 1974; Paomkki et al., 1987). The increase of algal growth usually occurs simultaneously with the decrease or disappearance of lichens. Bruck (1983) found that the abundance of algae increases on the periphery of a city, but diminishes towards the centre of the city. Thus, the abundance first increases towards polluted air, and then decreases again in the most polluted areas.

The use of epiphytic algae as bioindicators of air quality has been unusable in the past because of the lack of a suitable measuring method. The algae grow on tree trunks as diffuse layers covering the whole trunk. Only the thickness of the layer varies. Thus the point frequency methods used on lichens cannot be applied to algae. Bruck (1983) developed an index based on visual estimation and Turner (1985) used a laborious method of removing algae manually and then weighting their mass and spectrophotometrically analyzing the amount of chlorophyll. In order to use the algae as bioindicator of the air quality, the gradients of algal abundance must be found. This means that large amounts of point data must be collected. In urban areas, it is usually impossible to use data collection methods that does not damage or harm the trunks.

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Bioremediation of toxics in environment by bioaccumulation into yeast cells

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For toxics bioremediation in environment present nonbiodegradable substances an important problem. For such toxics, a good way of elimination could be a biological accumulation. Biodegradation was the first biological elimination way to be experimented. The studies have been made with selected bacteria but the results are not so efficient for several xenobiotics like PCB's: their biodegradation is significant only for the less chlorinated congeners and the most important part of PCB in environment are electric transformer fluids which are highly chlorinated mixtures.

For other toxics like heavy metals the only way of biological elimination is the accumulation and we compared those two phenomena on yeast cells. For bioaccumulation, microorganisms pick up toxics as xenobiotics or heavy metals without degradation and stock it. These phenomena need a structure able to make interactions between microorganisms and PCB's and this structure depends on the chemical composition of cell envelopes. We can modify this composition by culture medium modification for an optimization of this bioaccumulation.

In this work, we have studied the effects of cell wall modification for two yeast strains : *Rhodotorula glutinis* and *Saccharomyces cerevisiae* on PCB's bioaccumulation for an economical remediation of those substances from the environment.

¹K. POSTAL and ²G. FÜLEKY

**Direct and indirect effect of phosphorus supply
on mycorrhizal formation by *Glomus mosseae*
(Nicol. Gerd.) Gerdemann and Trappe on maize**

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The effects of vesicular-arbuscular (VA) endomycorrhizae, soil phosphorus amendments and root density were studied on the root colonization and phosphorus uptake of maize plants under greenhouse conditions. Maize plants were grown for six weeks in a pot experiment using calcareous sandy soil and three phosphorus rates (P_0 , P_{50} , P_{250}) varying from deficient to supraoptimal for the plant. The soils were either inoculated with VA endomycorrhizal fungi (MO+VA), or the plants were raised without VA inoculation (MO-VA). The various root densities were created by using pots with different volumes (0.5, 2.0, 5.0 and 10 litres).

The positive effect of mycorrhizal inoculation on the shoot growth declined as phosphorus fertilization was increased. For the various soil volumes, the effect of mycorrhizal inoculation on the shoot mass rose as the soil volume increased. In all cases, mycorrhizal plants had higher P contents. The positive effect of mycorrhizal inoculation on plant phosphorus uptake was exhibited even when phosphorus fertilization was applied. Phosphorus fertilization reduced the degree of root colonization and the quantity of external hyphae also. Our results on mycorrhizal growth effects have important implications. 1) It is difficult to make comparisons between pot experiments, because the size of reported mycorrhizal response tell us little beyond showing that a mycorrhizal response is possible under the growing conditions used. 2) The effect of phosphorus fertilizer on mycorrhizal infection appears to be mediated by the plant at low and medium levels of soil P, whereas soil P at high levels.

T. SZILI-KOVÁCS, F. GULYÁS, A. ANTON, and B. BITÓ

**Application of some soil biological methods
for the indication of the soil environmental quality**

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Soil biological effects of certain anthropogenic pollutions were investigated in arable soils in the frame of an international research project. The aim of the study was to develop and evaluate methods which were applicable to recognize unfavourable change in the soil state in time when the degradation process can still be stopped and reversed. First of all the effect of accumulating heavy metals and slowly degrading organic pollutants on the population and activity of soil microorganisms were investigated. The reason for using

these methods was supported by the fact that significant changes should be observed in the soil microbial population and in the dynamics of the most important transformation processes (carbon and nitrogen) having key roles in the soil functions.

Experiments were carried out using soil samples from the top layer of arable land near Százhalombatta and Visonta as the effect of airborne soil pollution caused by power plants. Oligotrophic and nitrogen-fixing bacteria were the most sensitive groups of the eight investigated bacterial populations. The most sensitive activity parameters were the dehydrogenase and nitrogenase activities. More CO₂ evolved and less biomass-C produced as the effect of soil pollution. Application and evaluation of the results were highly affected by the seasonal changes of the investigated soil biological parameters which should be considered.

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**Identification of some *Ornithobacterium rhinotracheale*
strains on the basis of their morphological, biochemical
and serological characteristics**

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Bacterial strains were cultured from the respiratory tract of 3 to 8 week old chicken and turkeys suffering from acute respiratory disease due to suspected *Ornithobacterium rhinotracheale* infection. Seventeen isolates were obtained and examined of which 12 originated from chicken and 5 from turkeys. The strains were identified on the basis of their cultural, morphological and biochemical characteristics, compared to a reference *O. rhinotracheale* strain (LMG 9086). Serological assignment was attempted by the tube agglutination test and by the passive haemagglutination test using immune sera produced in rabbits against the reference strain and four field isolates.

All isolates grew well on cooked blood agar medium, under microaerophilic conditions in the presence of 10% CO₂, after 48 h incubation at 37 °C. They formed tiny, wet, glistening, colourless, smooth colonies with entire edge. Strains could be isolated from chicken or turkeys only immediately after their death and not from carcasses sent for routine post mortem examinations.

Microscopically the bacteria appeared as 2 to 3 µm long rods, along with some longer filaments and often showed bipolar staining. All isolates were catalase negative, oxidase-positive and fermentative in the OF test. They were positive for urease, Voges-Proskauer, ONPG (beta-galactosidase), arginine dihydrolase, phosphatase, pyrazinamidase and hyaluronidase but gave negative reactions for indole, methyl-red, nitrate reduction, esculin hydrolysis, phenylalanine deaminase, gelatinase, arysulphatase, lecithinase and lipase. Acid was produced from glucose, lactose, mannose and wheat starch but not from sucrose, trehalose, sorbitol, mannitol, salicin and xylose.

Biochemically all isolates were identical to each other and to the reference *O. rhinotracheale* strain. All strains were sensitive to penicillin (except the reference strain), ampicillin, erythromycin, chlor- and oxytetracycline, ofloxacin and nitrofurantoin but were resistant against streptomycin, neomycin, gentamicin and polymyxin B. Serologically, all strains examined until now, along with the reference strain, belonged to a single serogroup using the tube agglutination test. By the passive haemagglutination test the strains could not be assigned.

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Effect of some xenobiotics on the growth of *Escherichia coli*, *Rhizobium meliloti* and *Rhizobium leguminosarum* strains

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Continuing previous plasmid transfer investigations carried out with *Escherichia coli* we studied the antibacterial effect of zinc-ethylene-bis-dithiocarbonate, manganese-zinc-ethylene-bis-dithiocarbonate and Hg^{2+} , Pb^{2+} , Cu^{2+} containing inorganic compounds on different strains of *E. coli*, as well as diazo-trophic *Rhizobium meliloti* and *Rhizobium leguminosarum* species. The MICs were determined by macrodilution method.

The strains tested proved to be highly resistant to zinc-ethylene-bis-dithiocarbonate and manganous-zinc-ethylene-bis-dithiocarbonate (MIC > 2000 µg/ml). The MIC and MBC represented very low values in the presence of Hg^{2+} (MIC=4–9 µg/ml). On the basis of MIC values a moderated level resistance was shown in the case of Pb^{2+} and Cu^{2+} (MIC=200–420 µg/ml).

It has been concluded that strains of *E. coli*, *R. meliloti* and *R. leguminosarum* tested were tolerant to a slight extent against Hg^{2+} content compounds.

I. F. KISS, K. KLÓSZ, J. FARKAS, and A. SZABÓ-SIMON

Estimation of viable cell count by bioluminescence method

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The microbiological control of processing and microbiological quality of products, the hygienic condition demand very quick methods in the food industry. One of them is the determination of ATP content of microorganisms from which the microbial load can be estimated. Living cells contain ATP and its quantity is relatively constant. The assay is based on the reaction of enzyme-substrate complex (luciferase–luciferin) and ATP, and it

produces light emission. The emitted light is directly proportional with the ATP concentration and the viable cell count.

In our experiments the microbiological contaminations of chicken meat, pork, sausage, spices and different surfaces in processing line of the poultry slaughterhouse (plucking, cutting, etc.) were studied. The calibration was carried out with isolated bacterial strains of meat-industrial origin and with microflora suspension from poultry carcasses by spiral-plate method. The bioluminescence technique can substitute well the spiral plate technique. Over 40 data the correlation coefficients were high (P 99.9%). The lower limit of detection of bacterial contamination is approx. $10^2 \text{ cm}^{-2} (\text{g}^{-2})$ by luminometry. The technique gives quick information on the microbial load. The results are available within 30 min.

¹R. KISS and ²G. SZITA

Use of Z-agar for the isolation of *Pseudomonas aeruginosa* and other *Pseudomonas* strains from food

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Beside the well known *Salmonella*, *Shigella*, *Yersinia*, *Escherichia coli*, *Campylobacter*, and other enteropathogenic bacteria also *Pseudomonas* strains may cause enteric diseases. In food-processing environments, *Pseudomonas* can contaminate the surfaces proliferate and may form biofilms. Using the HACCP system – in the course of food examinations and the control of the environment, isolation of facultative pathogenic *Pseudomonas* strains becomes improves by the use of Z-agar (Szita, Reanal). Z-agar contains acetamide as sole carbon source. As *Enterobacteriaceae* strains can not use acetamide as carbon source, we introduced the medium for the examination of *Pseudomonas* contamination of food.

For controlling selectivity, oxidase positive and oxidase negative bacteria – *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Citrobacter freundii*, *Proteus vulgaris*, *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Aeromonas caviae*, *Aeromonas veronii*, *Aeromonas schubertii* strains were cultured on Z-agar. Incubation was performed at 37 °C for 48 h. The above listed bacteria did not form colonies on Z-agar. Parallel to Z-agar the following media were used: GSP agar (FLUKA), eosin-methylene blue agar, brilliant-green agar, and blood agar.

After 24 h incubation *Pseudomonas* form small, circular, round colonies on Z-agar. *P. aeruginosa*, *P. putida*, *P. syringae*, *P. fluorescens* show fluorescence in UV light – after 48 h, also the medium itself becomes green.

In the course of the examination of samples of sea and fresh-water fish, crustaceans, poultry, sheep's cottage cheese, deep-frozen vegetables, lettuce, spices, dried fruit, the following strains were isolated: 41 *P. aeruginosa*, 1 *P. diminuta*, 3 *P. fluorescens*, 4 *P. mendocina*, 3 *P. putida*, 1 *P. stutzeri*. Using Z-agar – in comparison with using the other media – positivity increased by 20%. On other media, *Pseudomonas* colonies were often covered by colonies of other microbes or haemolysis disturbed the differentiation of

the colonies, thus false negativity was observed. Z-agar also helped the differentiation of *Pseudomonas* and *Aeromonas* strains, because of the parallel use of GSP-agar. Thus, from 5 samples, isolation of both species succeeded. The use of the inexpensive minimum medium Z-agar provides considerable benefits.

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Food-microbiological examinations completed by filtration technique for the detection *Campylobacter*, *Helicobacter* and *Arcobacter* species

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The microbiological investigation of *Campylobacter* started in 1980, the epidemiological surveillance activity became uniform and general in Hungary in 1990, on the basis of laboratory diagnostics performed by the Public Health Service. The notification of this infection is at present not yet obligatory. On the base of laboratory investigations and surveillance activity *Campylobacter* became the second frequent isolated enteropathogen organisms after the *Salmonella* in Hungary. About 90% of the strains are isolated from patients every year. The maximum of age – specific incidence rate (per 100 000 inhabitants) occur in children under 1 year, reaching more than 100 cases in the nineties. In the population under 5 years of age the *Campylobacter* infection play the leading role.

Campylobacter jejuni predominated in both sporadic and epidemic cases, but outbreaks are rare.

According to recent literary data, increasing number of *Helicobacter* and *Arcobacter* strains may be responsible for enteric diseases, we wished to complete our examinations by the use of filtration method, and incubation at different temperatures at microaerobic, aerobic and under anaerobic conditions.

Fifty raw chicken skin samples – from the production line in a slaughter-house, the use of HACCP system, 10 Danube samples (100 ml) were filtrated by the use of 0.45 µm pore size cellulose-nitrate membrane filters. The swabs and the filters were cultured in Preston enrichments incubated for 48 h at 25, 37 and 42 °C. After enrichment 0.45 µm pore size cellulose-acetate membrane filters were put onto the surface of Skirrow CCDA agar plates for the active migration of microbes. Aliquots of 0.1 ml from enrichment broth was dropped onto the membrane for the sake of active migration for 60 min. After this time the filters were taken off and the plates were incubated microaerobically, anaerobically, and aerobically at 42 °C, 37 °C and 25 °C for 48 h and 5 days. From the suspected *Campylobacter*, *Helicobacter*, *Arcobacter* colonies subcultures were made. The isolated strains were identified, serotyping were performed by the use of Lior sera-produced by us.

The food-microbiological examinations completed by filtration method were useful. Danube and raw chicken skin samples yielded the following strains: 75 *C. jejuni*

C. jejuni ssp. *doylei*, 51 *C. coli*, 6 *C. lari*, 2 *C. lari* ssp. UPTC, 2 *C. upsaliensis*, 5 *H. cinaedi*, 2 *H. fenneliae*, 1 *H. pylori* (Danube), 1 *A. caciaerophilus*, 3 *C. fetus*. In the water samples the number of the isolated strains were between 2 and 5. In 20% of the chicken samples the number of the isolated stains were 2 or 3 (different species and/or serotypes).

The serotypes were LIO 1, 2, 4, 6, 7, 8, 9, 11, 13, 15, 17, 19, 21, 22, 29, 35, 36, 38, 44, 45, 46, 48, 49, 50, 52, 53, 54, 56, 57, 59, 60, 63. The underlined serotypes were isolated from both chicken and water samples. Negative samples were found only at the end of the production-line.

With this method – comparing with conventional striking method – the % of positivity increased with 33%.

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Listeria monocytogenes in a small confectioner's workshop

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Between the years of 1994-1996 777 samples were examined for listeria contamination, and all proved to be negative. In this period, products contaminated with *Listeria monocytogenes* were found twice in the course of routine central of small confectioner's workshop. The first *Listeria* colonies were detected in both cases when examination was performed after enrichment in rhodanide broth for the detection of *Staphylococcus aureus*. The examined samples were the following: in 1994 ice-cream and in 1996 pastry with fruit, yoghurt and whipped cream. After this, comprehensive examinations started in the producing workshop and in the confectioner's shops where the products have been transported and saled. The followings were examined: all products, the environment at the production, transportation, serving; throat, hand, faeces and vaginal samples were taken from the producing persons. The Public Health Service checked at the same time the different spots and informed the interested physicians, calling attention to follow the condition of endangered persons – pregnant women, small children – and to inform the population about the possible risk of consuming pastry prepared with milk products and ice-cream. The immediate dosing and disinfectant cleaning of the workshops and confectioner's shop were measured. The repeated functioning was allowed only after having negative examination results.

The examination of the first samples were carded out according to the Standard of Ministry of Welfare 9/1986.(IX.17.) completed in 1995. The consecutive examinations were performed according to the ISO/DIS 11290-1,-2. Microbiology of food and animal feeding staffs – horizontal method for the detection and enumeration of *Listeria monocytogenes*. Fraser broth (Sanofi Diagnostice), Oxford and PALCAM agar (MERCK) were applied. Serotyping was performed with sera, produced by us and checked in inter laboratory trial by the WHO Listeria Workshop. In the first case in 1994, 7 ice-cream samples (vanilla, chocolate), the rinsing plastic tube of the feeding spoon and cooking pot were contaminated by *L. monocytogenes*. The serotype of the strains were 1/2a and 4b. In

the second case 2 cakes, 1 chocolate ice, cream, 2 whipped cream, one cream-can twice after the cleaning, 1 hand sample of a worker's, 1 faeces sample of an other workers were contaminated. The serotype of these stains was 1/2a. By reporting our present results, we wish to call attention for the use an efficacious cleaning method, and to call attention for the risk of confectioner's application of raw dairy products because they can be contaminated by *L. monocytogenes*.



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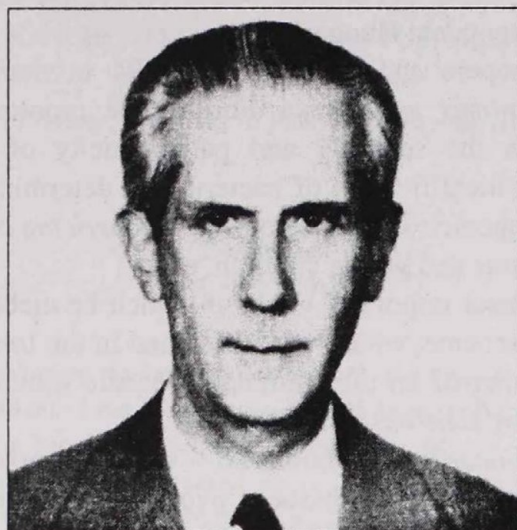
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PROFESSOR BÉLA LÁNYI

(1927–1997)



The microbiological research and practice in Hungary was afflicted by an irreparable loss early in this year: Professor Béla Lányi, editor of this periodical for more than 20 years, died after a long and painful suffering on January 11. His inexhaustible activity and his grounding in science and languages played a prominent role in that our *Acta* was able to retain its international esteem in the hard times when a majority of the sister journals had to cease publication.

B. Lányi was born in Budapest. He qualified in 1951 as M.D. at the University Medical School, Budapest. Four months later, he was admitted to the National Institute of Hygiene, Budapest, where he was active without interruption at the Department of Bacteriology until the end of 1996, when he retired. During the last 10 years, he headed the department.

His routine task included, in addition to the everyday epidemiological-clinical bacteriology, continuous improvement of the working conditions, methods, culture media, etc., to keep bacteriology at an up-to-date level not only in the central institute but also at the 20 public health stations of Hungary. From 1984 on, as the president of the Microbiological Expert Committee of the Ministry of Health, he introduced these activities in institutes of medical care and prevention including university laboratories. He organized a nation-wide supervision and external quality control of clinical bacteriology laboratories, and elaborated a unique survey system of bacteriological investigations, incidence and antibiotic resistance of the most important pathogenic bacteria.

With special regards to *Enterobacteriaceae* infections, he gradually organized a laboratory aimed at providing bacteriological laboratories in Hungary with modern diagnostic sera. In 1962, he was commissioned to reorganize the institute's culture collection, which in his hands developed into an internationally highly esteemed national collection of bacteria of medical importance.

He edited methodical guidelines and a handbook, each valuable for those who, being active in epidemiological and clinical bacteriology, use standardized methods. He was contributor of *Pharmacopoeia Hungarica*.

In his scientific papers and book chapters, 94 in number, the main subjects grouped around *Pseudomonas aeruginosa*. Further, he reported on the incidence of *Salmonella* infections, on the serology and pathogenicity of the *Proteus* group, on procedures to be used for identification of bacteria and determination of their sensitivity to antibiotics, on the pathogenicity of the serogroup *Escherichia coli* 0124, on the etiology of gastroenteritides of infants and young children, etc.

It was one of his most important works in which he elaborated a new system for *Pseudomonas* antigens, a scheme, which was welcomed in the International Nomenclature Committee. He was member of an international scientific group that was established to unify the different antigenic schemes of *P. aeruginosa*.

To refine *Pseudomonas* investigation, he – with co-workers – combined his own serological scheme with typing on the basis of pyocin production of bacteria. Thus, they defined 165 intraspecies types. They defined the antigenic relations between the *Enterobacteriaceae* family and *P. aeruginosa*. In co-operation with American researchers, Lányi described the antigenic scheme of a facultatively pathogenic *Pseudomonas* group. Based on his earlier antigenic scheme, his team studied the immunochemical properties of *P. aeruginosa* in detail. They determined the basic components and the immunoelectrophoretic properties of the somatic antigens, and defined the R antigens. They classified the factors inhibiting the O agglutination of *Pseudomonas* and other Gram-negative bacteria. They clarified the essence of polyagglutinability and induced this phenomenon by using phages. They examined the importance of *Pseudomonas* bacteria in water hygiene and dealt with the possibilities of protection against *P. aeruginosa*. With theses entitled "Characterization of *P. aeruginosa* from serological, hygienic and clinical views" he won the degree doctor scientiae medicinalis from the Hungarian Academy of Sciences. He co-operated with prominent Russian research institutes on the immunochemical structure of the *P. aeruginosa* O antigen.

In the Hungarian Society for Microbiology, he was a member of the governing body, chairman of the bacteriological section and a representative of the Society at the International Nomenclature Committee. Besides, he was member of several further committees and colleges.

Professor B. Lányi was owner of valuable titles and decorations.

On behalf of the Editorial Board

Dr E. Farkas

BIOPRODUCTION OF INDOLEACETIC ACID BY A *RHIZOBIUM* SP. FROM THE ROOT NODULES OF *DESMODIUM GANGETICUM* DC.

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The *Rhizobium* sp. isolated from the root nodules of *Desmodium gangeticum* DC. produced a high amount of indole acetic acid (IAA) from tryptophan in culture. For maximum IAA production, the bacteria preferred L-isomer over DL- or D-isomer of tryptophan. The production of IAA could be increased up to 37% over yeast extract ribose medium by supplementing the medium with ZnSO₄ (0.1 µg/ml), asparagine (0.1%) and nicotinic acid (0.1 µg/ml). The possible relationship between the rhizobial IAA production and legume-rhizobia symbiosis is discussed.

The formation, development and function of the root nodules of leguminous plants have remained a point of interest, a subject of controversy and of extensive research for a long time. The nitrogen supply was initially thought to be the only function of rhizobial root nodules. But later *Rhizobium*-derived hormones drew great attention to the scientists due to their involvement in the formation [1] and development [2] of the nodules. Among the phytohormones IAA controls almost all the physiological activities in plants. There are substantial evidences for the production of IAA in culture by *Rhizobium* spp. But most of the information came from the *Rhizobium* spp. isolated from the root nodules of agriculturally important pulses [3, 4]. Reports on the non-agricultural herbs are very scanty. *Desmodium gangeticum* is an unexplored and neglected leguminous herb. The plant is suitable for paper-making. The roots are used as febrifuge, expectorant and diuretic [5]. The nodules of *D. gangeticum* contained a moderate amount (4.5 µg/g fresh tissue) of IAA (data to be published elsewhere). There is no information of the ability of IAA production by its symbiont. But such information may throw some new light on the better understanding of the symbiosis of the root nodules of this plant. This investigation resulted in some interesting results which would help to have an insight on the legume-rhizobial symbiosis.

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Materials and methods

Organism, medium for growth and measurement. The symbiont was isolated from the fresh, healthy and mature root nodules of *Desmodium gangeticum*, and was identified as a species of *Rhizobium* following Jordan [6]. The basal medium for incubation was yeast extract mannitol medium of Skerman [7] with slight modification (0.01% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ instead of NaCl and CaCO_3 at pH 6.8), and supplemented with different isomers of tryptophan. The bacteria were incubated in 20 ml of medium in 100 ml Erlenmeyer flasks with three replicates at $30 \pm 2^\circ\text{C}$ on a rotary shaker to study the IAA production. Bacterial growth was measured turbidimetrically by an EEL (Evans Electroselenium Ltd., U.K.) colorimeter at 540 nm.

Estimation of IAA production. To estimate the IAA production, the medium was centrifuged at 5,000 g after the incubation time and the cell free supernatant was used for IAA production following Sinha and Basu [8]. To the supernatant equal volume of 1 N NaHCO_3 solution was added, then acidified with 9 N H_2SO_4 to pH 3.0. The mixture was extracted thrice, each time with equal volume of chilled, peroxide-free diethyl ether. The pooled ether phase was evaporated, the residue was eluted with ethanol and subjected to thin layer chromatography (TLC) on Silica Gel G developing with isopropanol: ammonia: water (10:1.4:0.6, v:v:v). The R_f zone corresponding to that of authentic IAA (Sigma, U.S.A.) was eluted with ethanol, dried, dissolved in water and used for colorimetric estimation following Gordon and Weber [9].

Initially different carbon sources were added separately to the tryptophan supplemented mannitol-less basal medium. Then the different chemicals (except vitamins) were added individually to the tryptophan supplemented basal medium with most suitable carbon source and the individual effect of the chemicals on IAA production was checked. The vitamins were added to the tryptophan supplemented basal medium omitting yeast extract. To find out the maximum IAA production by the *Rhizobium* sp. in culture, the medium was enriched with the supplements which individually increased the maximum IAA production. All the chemicals used for cultural optimization were sterilized through bacterial filters and were added to the medium aseptically.

Statistical analyses were done according to Panse and Sukhatme [10].

Results

The isolated *Rhizobium* sp. produced IAA in culture when supplemented with tryptophan. The bacteria preferred L-tryptophan (1.5 mg/ml) for both growth and IAA production (Fig. 1). The growth phase and the production phase of the bacteria started simultaneously and reached their respective stationary phases in almost 36 h (Fig. 2).

The effects of carbon sources, minerals, nitrogen sources and vitamins on IAA production by this rhizobia were checked separately. The ribose (1%, Tables I and II), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1 $\mu\text{g}/\text{ml}$, Tables III and IV), asparagine (0.1%, Tables V and VI) and nicotinic acid (0.1 $\mu\text{g}/\text{ml}$, Tables VII and VIII) had the maximum effect in increasing IAA production. To check the maximum IAA production in culture, the chemicals which individually increased the IAA production to the most were added to the medium. The production of IAA was increased from 200.7 $\mu\text{g}/\text{ml}$ (in YE ribose medium) to 275.2 $\mu\text{g}/\text{ml}$ (Table IX).

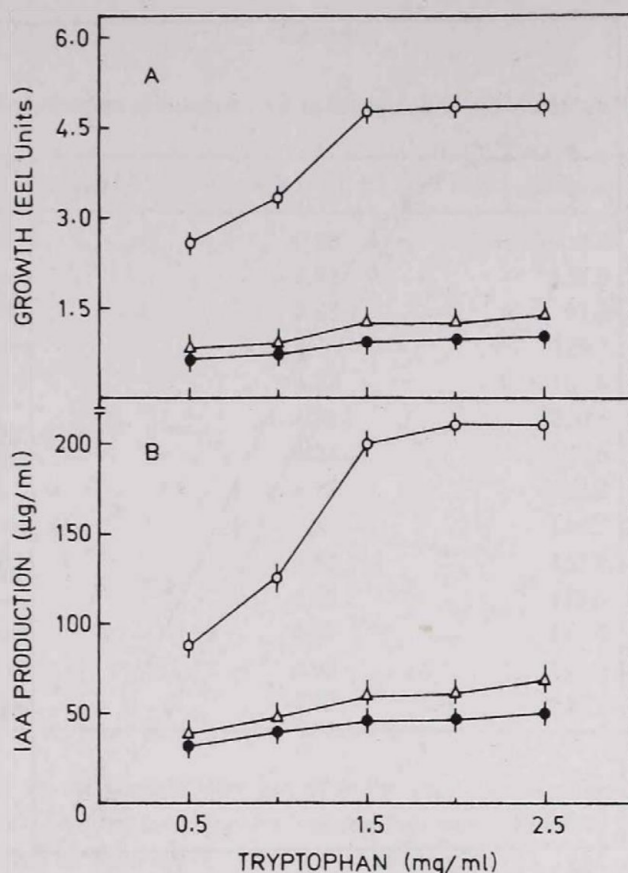


Fig. 1. Effect of L-tryptophan (○), DL-tryptophan (△) and D-tryptophan (●) on growth (A) and IAA production (B) by the *Rhizobium* sp. in yeast extract ribose medium.

The readings were taken after 36 h. Bars on the points indicate $\pm$ SE

Discussion

The *Rhizobium* sp. produced IAA in the culture when supplemented with tryptophan. The bacteria preferred L-tryptophan for both growth and IAA production (Fig. 1). Similar observations were made by Dullaart [3] and Chattopadhyay and Basu [11] for other species of *Rhizobium*. Toxic effect of D-tryptophan on growth of *Rhizobium* as reported by Clark [12] might be the cause of the lower IAA production and growth in D- and DL-tryptophan supplemented medium (Fig. 1).

Both growth and IAA production phase of the bacteria started immediately after inoculation and continued simultaneously (Fig. 2). Secondary metabolites are generally produced in the stationary phase of growth of bacteria. Production of this secondary metabolite, IAA, along with growth of this *Rhizobium* is an interesting feature (Fig. 2).

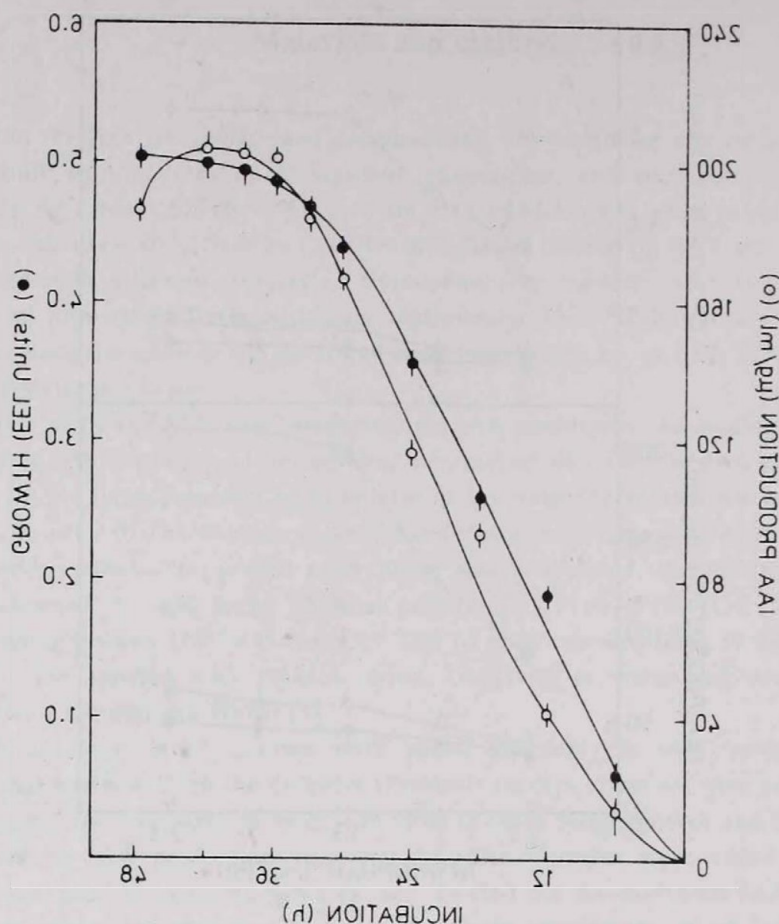


Fig. 2. Changes in growth (●) during IAA production (○) by the *Rhizobium* sp. in the L-tryptophan (1.5 mg/ml) supplemented yeast extract ribose medium.
Bars on the points indicate $\pm$ SE

Among the thirteen carbon sources tested (Table I), all the carbon sources (at 1% level) promoted both growth and IAA production to different extent (Table I) and among these, ribose (1%) was found to be the most preferable for better IAA production (Tables I and II). Yoshida and Yatazawa [13] reported earlier that carbon source played a key role in the growth and IAA production of *R. trifolii*. Thus carbon source can be regarded as one of the determining factors for IAA production by the symbiont.

Minerals had some promotive effect on nitrogen fixing capacity of *R. japonicum*. [14]. This led to test the effect of minerals on the IAA production. Among the six metal ions tested, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1 µg/ml) was the most suitable for better IAA production (Tables III and IV). The promotive effect of Zn^{2+} on IAA production might be due to the better growth of the bacteria (Table III). Though in this case, better growth was not the only cause for better IAA production as maximum growth was obtained in CoCl_2 .

Table I*Effect of different carbon sources on IAA production and growth by the Rhizobium sp.*

Carbon sources (1%)	Growth (EEL Units)	IAA production (µg/ml)
Control	0.80	14.9
Glucose	3.92	138.9
Inositol	2.83	97.2
Arabinose	4.76	178.1
Maltose	3.82	103.6
Ribose	4.85	200.8
Fructose	4.23	134.6
Xylose	4.72	152.2
Mannose	4.58	141.2
Lactose	4.82	155.4
Sucrose	4.65	117.9
Raffinose	4.63	110.4
Galactose	4.90	182.9
Mannitol	4.67	140.3

In control set the bacteria were grown in the yeast extract mineral medium containing 1.5 mg/ml L-tryptophan. The readings were taken after 36 h which was optimum for maximum IAA production (Figure 2)

Table II

Effect of different concentrations of the most preferred carbon source (ribose) on IAA production and growth of the bacteria

Concentration (%)	Growth (EEL Units)	IAA production (µg/ml)
0.5	4.52	170.4
1.0	4.84	200.7
1.5	4.79	198.7
2.0	4.65	188.4
3.0	4.58	181.2

The bacteria were grown in yeast extract mineral medium with different concentrations of ribose. Other conditions as in Table I

Table III

Effect of different metal ions on IAA production and growth of the Rhizobium sp.

Metal ions (as salts) (0.1 µg/ml)	Growth (EEL Units)	IAA production (µg/ml)
Control	4.84	200.7
Co (CoCl ₂ ·6H ₂ O)	6.82	212.6
K (KI)	5.00	210.2
Fe (FeSO ₄ ·7H ₂ O)	6.00	206.8
Cu (CuSO ₄ ·5H ₂ O)	6.03	214.8
Zn (ZnSO ₄ ·7H ₂ O)	6.06	235.7
Ni (NiCl ₂ ·6H ₂ O)	5.06	231.1

The control had yeast extract mineral medium with ribose.

Other conditions as in Table I

Table IV

Effect of different concentrations of the most preferred metal ion (ZnSO₄·7H₂O) on IAA production and growth by the Rhizobium sp.

Concentration (µg/ml)	Growth (EEL Units)	IAA production (µg/ml)
0.01	4.86	222.3
0.05	5.00	230.0
0.10	5.06	235.8
0.20	5.06	235.9
0.30	5.00	230.2

The bacteria were grown in yeast extract mineral medium with 1% ribose.

Other conditions as in Table I

Among the inorganic and organic nitrogen sources tested (at 0.1% level) asparagine (0.1%) was the most effective for better growth and IAA production (Tables V and VI). Vincent [15], Jordan [6] reported earlier that *Rhizobium* spp. could use several nitrogenous compounds for the growth, which might be one of the causes for increased IAA production. On the other hand, in the presence of amino acids like glutamic acid, alanine, the conversion of tryptophan to IAA by *R. meliloti* was inhibited [16].

Table V

Effect of different nitrogen sources on IAA production and growth of the Rhizobium sp.

Nitrogen sources (0.1%)	Growth (EEL Units)	IAA production (µg/ml)
Control	4.83	200.7
Glycine	2.29	155.9
Casamino acid	5.33	178.3
Asparagine	6.66	232.5
(NH ₄) ₂ SO ₄	4.55	169.1
KNO ₃	5.05	200.1
NaNO ₃	5.27	218.1
NaNO ₂	2.36	170.9
NH ₄ Cl	2.49	164.5

Filter sterilized nitrogen sources were added to the experimental sets.
Other conditions as in Table III

Table VI

Effect of different concentrations of the most preferred nitrogen source (asparagine) on IAA production and growth by the bacteria

Concentration (%)	Growth (EEL Units)	IAA production (µg/ml)
0.025	6.28	198.9
0.05	6.35	212.4
0.10	6.60	232.5
0.20	6.28	190.5
0.30	6.00	134.3

The conditions of experiment were same as in Table III

The production of IAA and growth was increased by some vitamins (Tables VII and VIII) suggesting that vitamins might have some role on the IAA production. Better growth of this bacteria in the presence of vitamins might be one of the causes of better IAA production. Graham [17], Alexander [18] also reported that some vitamins like biotin, thiamine and pantothenate are required for the growth of *Rhizobium* spp.

Table VII

Effect of different vitamins on IAA production and growth by the Rhizobium sp.

Vitamins (0.1 µg/ml, except biotin)	Growth (EEL Units)	IAA production (µg/ml)
Control	2.39	119.2
Riboflavin	2.47	110.3
Pyridoxine-HCl	2.00	110.3
Thiamine-HCl	2.39	174.1
Para-amino benzoic acid	2.43	136.0
Biotin (0.001 µg/ml)	2.49	145.1
Ca-pantothenate	2.57	168.3
Nicotinic acid	2.49	179.9

Filter-sterilized vitamins were added separately to the yeast extract free basal medium containing 1.5 mg/ml L-tryptophan. The control set contained no vitamin. Other conditions as in Table III

Table VIII

Effect of different concentrations of the most preferred vitamins (Nicotinic acid) on the IAA production and growth by the bacteria

Concentration (µg/ml)	Growth (EEL Units)	IAA production (µg/ml)
0.01	2.09	139.8
0.05	2.18	151.4
0.10	2.29	179.8
0.30	2.20	156.4
0.50	2.20	156.1

Other conditions of the experiment were as in Table VII

When the yeast-extract-ribose medium was supplemented with the chemicals which individually increased the IAA production to maximum, the production increased from 200.7 µg/ml to 275.2 µg/ml (an increase of about 37%) (Table IX). Thus, the composition of the medium plays a definite role in the IAA production by the *Rhizobium* sp.

Table IX

Increase in IAA production and growth by the Rhizobium sp. by the most effective supplements

Supplements	Growth		IAA production	
	(EEL Units)	Increase over control (%)	µg/ml	Increase over control (%)
Control	4.84	—	200.7	—
+ ZnSO ₄ ·7H ₂ O (0.1 µg/ml)	6.06	25.2	235.7	17.4
+ ZnSO ₄ ·7H ₂ O + Asparagine (0.1%)	6.60	36.4	255.9	27.5
+ ZnSO ₄ ·7H ₂ O + Asparagine + Nicotinic acid (0.1 µg/ml)	6.80	40.5	275.2	37.1

The control contained yeast extract ribose medium with L-tryptophan (1.5 mg/ml).

Other conditions as in Table I

The root nodules of *D. gangeticum* contained 4.5 µg of IAA/g fresh wt (data to be published elsewhere). The high amount of IAA produced by this *Rhizobium* sp. might be responsible for the nodular IAA. Evensen and Blevins [19] also reported that when they inoculated Limabean plants separately with two different strains of *Rhizobium* sp. different levels of GA were observed in the nodules. In this present investigation the supplements which increased the IAA production in culture might have been available for the bacteria within nodules for the increased IAA production. By such alteration in supply of metabolites within the nodules, the host plant might induce the symbiont to produce more IAA for its own benefit. Again the hormones viz. auxin [20], gibberellin [19] and cytokinin [21] might be transported to the other plant parts for metabolism. So, the root nodules of this plant may also serve as a good source of phytohormones for the other plant parts as and when required. Hence the rhizobia-legume symbiosis needs a fresh review in the light of the nodular phytohormones; their production, metabolism, and supply to the host can be considered also as a vital aspect in the symbiosis along with nitrogen fixation.

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BIOSTATIC AND BIOCIDAL ACTIVITIES OF WATER-THINNABLE POLYESTERAMIDE COMPOSITIONS CONTAINING 8-HYDROXYQUINOLINE

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Various water soluble polyesteramide compositions are prepared by solvent technique containing a stoichiometric amount of 8-hydroxyquinoline as preservative against microbiological attack. Mechanism of action of 8-hydroxyquinoline against *Pseudomonas aeruginosa* ATCC 10145 is discussed and the studies showed promising results as biostatic and biocidal coatings. Preparation of water soluble polyesteramides via solvent process by using a new technique is another aspect taken into consideration.

Water-born coating are currently the subject of great interest, and they have many important new development [1–3].

Owing to its chemical composition and to the water present, water-born coating has a high nourishment value for fungi and bacteria and consequently is very susceptible to mold growth and bacterial degradation. The biodeterioration of paints fall into two basic categories: the enzymatic degradation of protein and cellulosic additives which produce a loss of viscosity in water-born coatings that are in liquid state, and the microbial deterioration of the applied film. In order to protect both the liquid paint and dried film against microbiological attack, compounds toxic to bacteria and fungi are added in the formulations. The predominant organism associated with paint films, *Pseudomonas aeruginosa*, is responsible for the deterioration of most latex paints in storage. A much greater number of bacteria and fungi are found in applied film and the

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most predominant fungi is *Pullularia pullulans* [4]. Others found were *Cladosporium* sp., *Alternaria dianthicola* and *Phoma pigmentivora* [5]. The microflora which grows on interior paints consists mostly of the *Aspergillus* and *Penicillium* species [6].

Various chemical agents are used to control or prevent the deterioration by microorganisms, zinc metabolites being examples of the inorganic pigments used for this purpose. Organomercurials and halogenated phenols are examples of the organic additives used for the same purpose. Their activity is based primarily upon their interference with metabolic functions of the bacteria and/or fungi [7, 8].

Microbiological contamination can originate from a number of sources including water, raw materials, manufacture plant, and the final container. Powder raw materials, such as fillers, extenders and pigments, especially those originating from natural sources, will often be contaminated with the spores of bacteria and fungi. Growth of microorganisms in the container after filling can also occur if the container is not biologically clean as a result of storage under unsuitable conditions [9, 11].

The aim of this work was to investigate the possibility of applying a new preservative agent of wide spectrum against bacteria and fungi as additive for water-born formulations.

Materials and methods

8-Hydroxyquinoline: chemically pure grade, El-Nasr Pharmaceutical Chemical Company, Egypt (m.p. 72.5–76 °C). The minimum inhibitory concentration (MIC) was determined for each test organism used individually. MIC, 2×MIC and 4×MIC were for applied *Pseudomonas aeruginosa* ATCC 10145.

Bacteria and fungi. Bacteria employed were *Bacillus subtilis* NCTC 8236, *Bacillus pumilus* NCTC 8241, *Micrococcus luteus* ATCC 9341, *Staphylococcus aureus* NCTC 7447, *Escherichia coli* BPPO1, *Pseudomonas aeruginosa* ATCC 10145 and *Klebsiella pneumoniae* NCIB 9111; *P. aeruginosa* was used for mode of action study, while fungi were *Candida albicans* IMRU 3669, *Saccharomyces cerevisiae*, *Aspergillus fumigatus*, *A. niger*, *A. terreus* F., *Penicillium citricus* and *P. citricus* F. (fungal isolates were provided by the mycology laboratory, Faculty of Sciences, Al-Azhar University).

All other solvents and chemicals used throughout the investigation were of the purest grade available, except where mentioned.

Viable cell count. An overnight culture of *P. aeruginosa* was reinoculated into fresh medium in a series of flasks prepared by addition of graded amount of 8-hydroxyquinoline and incubated for a period of 6 h. Samples for viable cell counts were removed from all flasks after every two hours of incubation. Viable cell count were determined by dilution of the culture using sterile medium at room temperature and by spreading 0.1 ml sample on the surface of nutrient agar plates. The colonies which developed were counted after 24 h of incubation at 30 °C.

Effect of 8-hydroxyquinoline on the synthesis of macromolecules in *P. aeruginosa*. Samples (15 ml) of the treated culture were chilled, centrifuged and washed with cold distilled water, the washed cells were extracted with 0.5 ml of 0.25 N perchloric acid at 70 °C for 15 min. The extract was used for estimating DNA by diphenylamine reaction [12], and RNA content was measured using orcinol reaction [13]. The pellet in each centrifuge tube after extraction of DNA and RNA with perchloric acid was dissolved in 2 ml N sodium hydroxide at 90 °C for 30 min and the protein content was measured by the Folin-phenol reagent according to Lowry et al. [14].

Methods of preparation: Preparation of hydroxyethyl fatty acidamides of linseed oil (HELA) [15]. Hydroxyethyl fatty acidamides of linseed oil (HELA) was prepared from diethanolamine (11.03 g, 0.105 mole), linseed oil fatty acids (0.1 mole), zinc oxide (0.122 g, 0.0015 mole) and xylene (15 ml) using Dean and Stark apparatus. The reaction was continued till 1.8 ml of water (0.1 mole) was liberated.

Preparation of water-soluble polyesteramides via solvent process. A mixture of calculated amounts of pentaerythritol, hydroxyethyl linseed oil fatty acidamides (HELA), and phthalic anhydride were refluxed in xylene solvent (10 ml) until approximately half the theoretical amount of water was collected (Table I).

To the resin, (10 ml) 2-butoxyethanol was added with good stirring, then the product was neutralized with ammonia to pH 9. The solid content was adjusted by using water/ethanol mixture (9:1) by volume.

Table I illustrates the resin characteristic constants and weight charges of various water soluble formulations. These formulations cover a wide range of various excess hydroxyl content (0, 10, 20 and 30% excess hydroxyl, respectively). The following abbreviations were used: HELA: hydroxyethyl linseed oil fatty acidamides; PE: pentaerythritol; PA: phthalic anhydride; e_o : total equivalents present at start of reaction; E: equivalent weight; e_A : number of acid equivalent; e_B : number of hydroxyl equivalent; F: functionality; W: weight in grams; K: alkyd constant ($= m_o/c_A$); R: e_B/e_A .

Results and discussion

Solvent type coatings based on polyesteramide resins have been described [16, 17]. These types combine the useful properties of polyester and polyamide resins. Light coloured polyesteramides are recently prepared via solvent process [15]. The present work is target to prepare a water-soluble polyesteramide via solvent process using a new technique which involves the presence of xylene (azotropic solvent) and 2-butoxyethanol among the aqueous media.

After a successful preparation and solubilization, pentaerythritol modified polyesteramides. Various varnish compositions were prepared by thinning with water containing 10% ethanol to 50% solid content, and the following drier combinations were added; Co and Mn acetates 0.02 and 0.01% based on metal/solid resin, respectively. 8-Hydroxyquinoline was then added at five different concentrations (0.00, 0.02, 0.04, 0.10 and 0.20% based on solid resin) as shown in Table II.

Glass-tine plates and steel panel substrates were used according to the named experiment. These resin varnishes and their films were subjected to preliminary evaluation followed by subjecting to an extensive evaluation study to indicate the most suitable compositions. Methods of analysis, testing and evaluation used in this work were performed according to standard methods [18–27]. The results are listed in Tables II and III.

From the data recorded in Tables II and III, the following conclusions were drawn.

1. Colour measurement of varnishes increases through the addition of 8-hydroxyquinoline.
2. Viscosity of varnishes show no significant effect by the presence of preservative 8-hydroxyquinoline.
3. Air drying and stoving time are slightly increased by increasing the amount of preservative.
4. The film performances and durability of various polyesteramide films are not affected upon the addition of 8-hydroxyquinoline.

Table I

Resin characteristic constants of various water soluble formulations

Resin No.	Excess -OH%	Ingredients	e_o	E	e_A	e_B	F	W_{gm}	m_o	K	R	H ₂ O off ml A. V		
												Ther.	Exp.	mg KOH/g
I	0	HELA	0.208	184.00	—	0.208	2	74.84	0.143					
		PE	0.052	34.04	—	0.052	4	1.77	0.018	1.00	1.00	2.3	1.15	51.3
		PA	0.259	74.10	0.259		2	19.19	0.149 0.310					
II	10	HELA	0.240	184.00	—	0.240	2	44.16	0.120					
		PE	0.060	34.04	—	0.060	4	2.04	0.015	1.04	2.4	1.2	1.2	60.4
		PA	0.273	74.10	0.273	—	2	20.22	0.136					
III	20	PE	0.072	34.04	—	0.072	4	3.06	0.018	1.10	1.20	2.6	1.3	61.3
		PA	0.298	74.10	0.289	—	2	20.88	0.149 0.310					
IV	30	HELA	0.351	184.00	—	0.351	2	49.31	0.143					
		PE	0.110	34.04	—	0.110	4	3.06	0.018	1.15	1.30	3.0	1.5	57.4
		PA	0.339	74.10	0.339	—	2	20.8	0.149 0.310					

Biological properties. Hydroxyquinolines were used for many years as an antiseptics before their amebicidal properties were discovered [28]. Subsequently [29–32] they were introduced as amebicidal agents. The main target of the present investigation is to use 8-hydroxyquinoline as preservative for water-based varnish compositions.

Table II

Effect of 8-hydroxyquinoline upon varnish characteristics

Resin No.	Excess-OH, 8-hydroxyquinoline, %	Excess-OH, 8-hydroxyquinoline, %	Colour "Gardner"	Viscosity "Gardner"	Air drying time** (h)	Stoving at 110 °C (min)
Ia	0	0.00	13	D	48	90
b	0	0.02	13	D	48	90
c	0	0.04	14	D	52	90
d	0	0.10	14	D	56	100
e	0	0.20	14	D	60	120
IIa	10	0.00	15	F	48	75
b	10	0.02	15	F	48	75
c	10	0.04	15	F	48	80
d	10	0.10	16	F	54	90
e	10	0.20	16	E	62	90
IIIa	20	0.00	13	E	40	80
b	20	0.02	13	E	40	80
c	20	0.04	13	E	42	85
d	20	0.10	14	E	46	90
e	20	0.20	14	E	48	100
IIa	30	0.00	14	H	36	70
b	30	0.02	14	H	38	75
c	30	0.04	15	H	38	75
d	30	0.10	15	H	40	85
e	30	0.20	15	G	45	90

* Based on solid resin

** Mean hard dry time

The activity of 8-hydroxyquinoline against bacteria, unicellular and filamentous fungi is given in Table IV. 8-Hydroxyquinoline showed a strong action against bacteria especially Gram-positive bacilli. Meanwhile filamentous fungi were more drastically affected by the antimicrobial agent than unicellular fungi. Furthermore, Elippoulos et al. [31] were able to select resistant colonies of *P. aeruginosa* ATCC 27853 at a relatively high concentration of the carboxyquinoline tested (64 µg/ml). In contrast, selected

colonies of *P. aeruginosa* ATCC 10145 are found to be capable of growing on 18 µg of 8-hydroxyquinoline per ml containing medium.

When tested in mice by intraperitoneal administration the compound was found to have an LD₅₀ of less than 17 mg/kg.

Effect of 8-hydroxyquinoline on cell morphology and viable cell count. The effect of 8-hydroxyquinoline on the morphology of *P. aeruginosa* cells was rather extreme. Cells elongate by an observable manner without separation into individuals in the presence of 8-hydroxyquinoline at 2×MIC for 2 h. This elongation apparently changed to cell disruption and lysis when the concentration of 8-hydroxyquinoline increased to 4×MIC for 6 h (Plate 1).

Table III

Film performances of various polyesteramide compositions

Resin	Hydrness		Flexibility	Gloss at		Adhesion	Resistance to							
No.	at 20 μ (sec)			60 °C			Water		Acid		Alkali		Solvent	
	a	s		a	s		a	s	a	s	a	s	a	s
Ia	104	180	Pass	84	80	Good	21	>30	20	>30	-	-	27	>30
b	111	173	Pass	84	84	Good	21	>30	16	>30	-	-	26	>30
c	107	165	Pass	97	86	Good	20	>30	20	>30	-	-	>30	>30
d	112	170	Pass	96	82	Good	19	>30	15	>30	-	-	>30	>30
e	102	153	Pass	97	84	Good	20	>30	15	>30	-	-	>30	>30
IIa	124	196	Pass	90	86	Good	23	>30	27	>30	-	1	>30	>30
b	114	160	Pass	94	85	Good	23	>30	>30	>30	-	1	>30	>30
c	100	178	Pass	90	85	Good	20	>30	>30	>30	-	-	27	>30
d	117	184	Pass	95	82	Good	24	>30	27	>30	-	1	27	>30
e	110	160	Pass	95	86	Good	20	>30	>30	>30	-	1	27	>30
IIIa	170	208	Pass	82	84	Good	27	>30	27	>30	-	-	>30	>30
b	160	190	Pass	90	82	Good	24	>30	25	>30	-	-	>30	>30
c	173	214	Pass	86	89	Good	27	>30	25	>30	-	-	>30	>30
d	156	194	Pass	92	88	Good	>30	>30	>30	>30	-	-	>30	>30
e	150	180	Pass	96	90	Good	>30	>30	>30	>30	-	-	>30	>30
IVa	182	224	Pass	84	80	Good	>30	>30	>30	>30	-	-	>30	>30
b	168	211	Pass	90	82	Good	27	>30	>30	>30	-	-	>30	>30
c	170	208	Pass	93	86	Good	>30	>30	27	>30	-	-	27	>30
d	168	204	Pass	90	86	Good	>30	>30	>30	>30	-	-	>30	>30
e	172	220	Pass	86	80	Good	25	>30	>30	>30	-	-	>30	>30

a and s mean air dried and stoved films, respectively

Flexibility and adhesion tests given for both air dried and stoved films

Resistance test given in days



Plate 1. The effect of applying 8-hydroxyquinoline on the cell morphology of *P. aeruginosa* ATCC 10145 (phase contrast photomicrograph). a=Control, $\times 1250$;
b=80 $\mu\text{g/ml}$ showing extreme elongation of cells, $\times 1250$

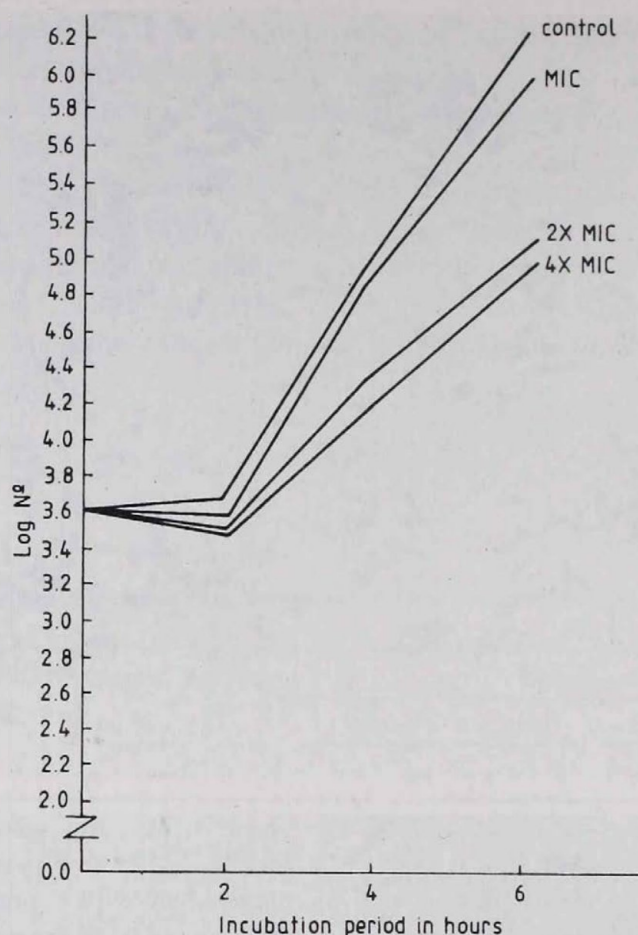


Fig. 1. Effect of 8-hydroxyquinoline on viable cell count of *P. aeruginosa* ATCC 10145

The effect of 8-hydroxyquinoline on viable plate count revealed morphological changes in *P. aeruginosa* cells. Indeed, viable plate count dropped down as the concentration and the incubation period (exposure of the antimicrobial agent) increased (Fig. 1). For instance, at 4×MIC after 2 h the log number of cells was 2.9. It decreased to nil at the same concentration after 6 h. The inhibition of cell division was supported by the lysis occurred at 4×MIC after 6 h.

Effect of 8-hydroxyquinoline on DNA, RNA and protein biosynthesis of P. aeruginosa ATCC 10145. The effect of 8-hydroxyquinoline on DNA, RNA and quantitative biosynthesis in *P. aeruginosa* was clearly observed. Worthy to mention that an increase in the amount of the antimicrobial agent was accompanied by a decrease in the macromolecular biosynthesis of *P. aeruginosa* (Figs 2, 3, 4). Probably the biosynthesis of protein as well as biosynthesis of macromolecules such as DNA and RNA was completely inhibited as the concentration of the 8-hydroxyquinoline had increased up to 4×MIC after 6 h.

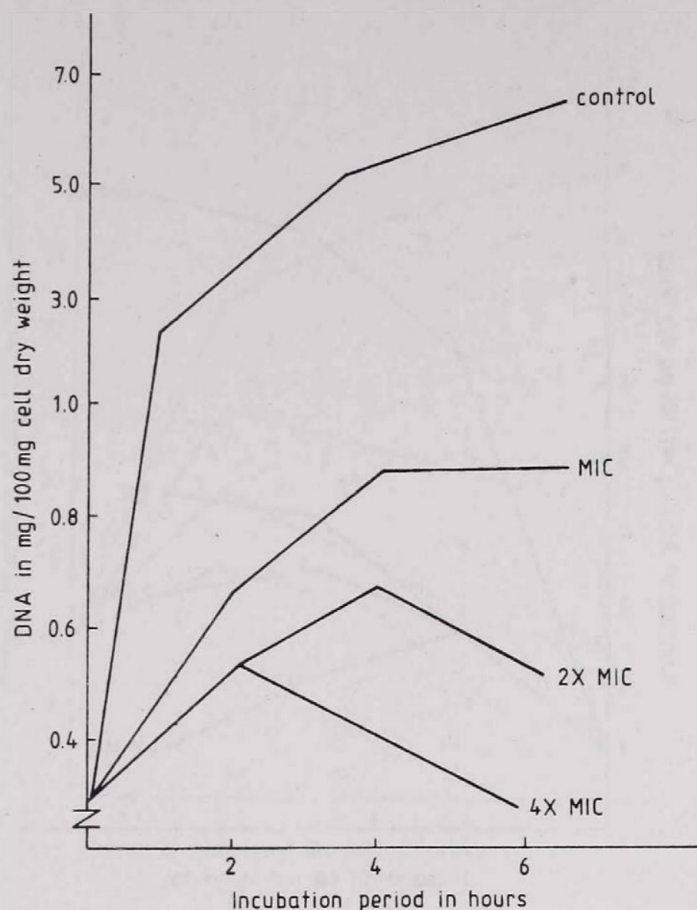


Fig. 2. Effect of 8-hydroxyquinoline on DNA biosynthesis in *P. aeruginosa* ATCC 10145

Protein biosynthesis is clearly associated with nucleic acids and the lack of protein biosynthesis may be a secondary effect of inhibition of nucleic acid biosynthesis. Quinoline compounds are known to affect DNA gyrase and DNA nicking-closing enzymes [32]. More recently, Hidaka et al. [33] reported that isoquinoline sulfonamides inhibit protein kinase C and cyclic nucleotide dependent protein kinases, competing with ATP. Now 8-hydroxyquinoline appears to be the most potent inhibitor of protein as well as DNA and RNA.

Alternatively, several antibiotics are known to inhibit protein biosynthesis without affecting nucleic acid biosynthesis. A partial list of these antibiotics include streptomycin and erythromycin [33].

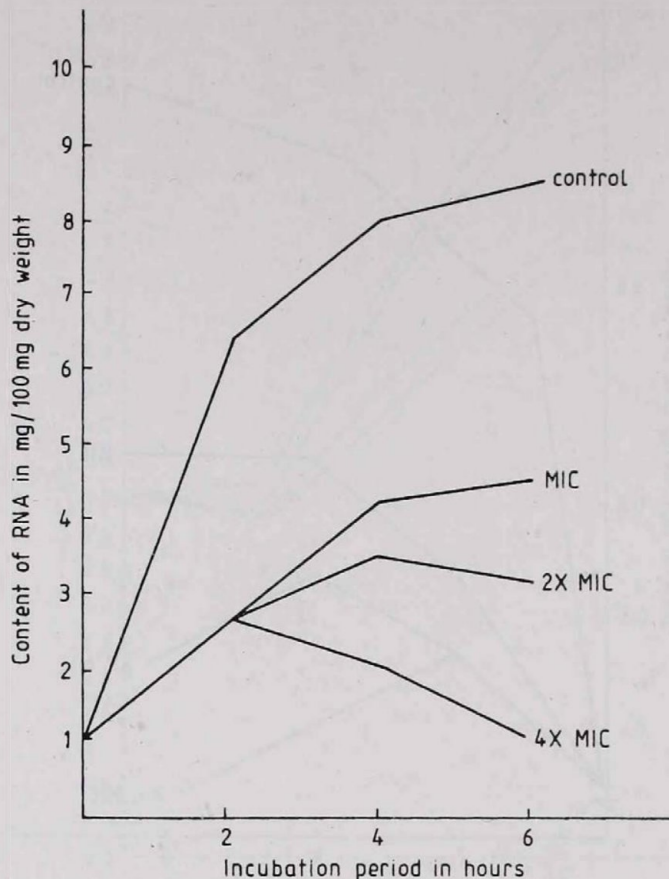


Fig. 3. Effect of 8-hydroxyquinoline on RNA biosynthesis in *P. aeruginosa* ATCC 10145

The 99% killing concentration was also determined proving that 8-hydroxyquinoline has a bactericidal effect on most of the Gram-positive bacteria.

Activity of polyesteramide compositions. The results in Table V show that polyesteramide compositions containing 8-hydroxyquinoline exerted marked inhibitory action against the tested bacterial strain as well as an effect on *Penicillium*, contrary to that unicellular fungi and *Aspergillus niger* which are unaffected by any composition of polyesteramide. More likely *A. fumigatus* and *A. terreus* F. are inhibited by polyesteramide composition No. I_{b,c,d,e}; II_{b,c,d,e}; III_{b,c,d,e}; and IV_{b,c,d,e}, respectively.

Polyesteramide compositions No. II_{b,c,e} and III_{b,c,e} (the only tested compositions) were strong inhibitors after 12 and 24 weeks against all tested bacteria and filamentous fungi (except *A. niger* and unicellular fungi). The results are consistent with that previously investigated on vinyl-type compositions containing pentachlorophenyl acrylates [35].

It is noteworthy to mention that different varnish compositions throughout the investigation had clear appearance and their solution in water/ethanol mixture were stable when stored in well-closed containers for more than six months except those of short-oil length (composition IV) which separated to two layers after more than four months.

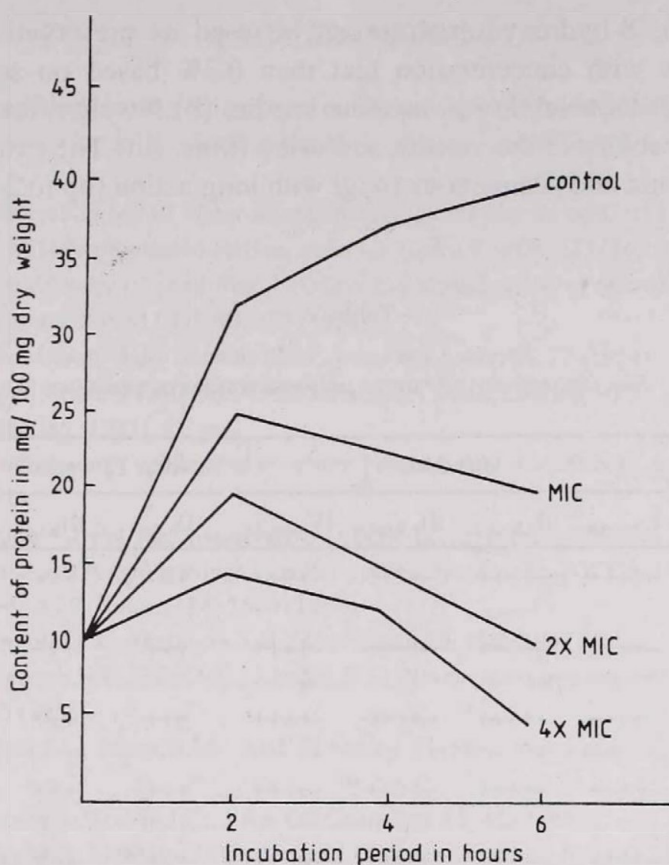


Fig. 4. Effect of 8-hydroxyquinoline on protein biosynthesis in *P. aeruginosa* ATCC 10145

Table IV

MIC of 8-hydroxyquinoline against bacteria and fungi

Organism	MIC µg/ml
<i>Bacillus subtilis</i> NCTC 8236	>5
<i>Bacillus pumilus</i> NCTC 8241	>5
<i>Micrococcus luteus</i> ATCC 9341	>5
<i>Staphylococcus aureus</i> NCTC 7447	10
<i>Escherichia coli</i> BPP01	15
<i>Pseudomonas aeruginosa</i> ATCC 10145	20
<i>Klebsiella pneumoniae</i> NCIB 9111	15
<i>Candida albicans</i> IMRU 3669	125
<i>Saccharomyces cerevisiae</i>	100
<i>Aspergillus fumigatus</i>	50
<i>Aspergillus niger</i>	80
<i>Aspergillus terreus</i> F	30
<i>Penicillium citricus</i>	20
<i>Penicillium citricus</i> F	20

In conclusion, 8-hydroxyquinoline can be used as preservative for water-born varnish formulations with concentration less than 0.2% based on solid resin, for the following reasons: (i) Its solubility in aqueous media. (ii) No significant effect upon the performance and durability of the varnish and dried films. (iii) The extreme effectiveness upon growth of bacteria and filamentous fungi with long action (up to 24 weeks).

Table V

Biocides activity of various polyesteramide compositions

Microorganism	After 24 h				After 12 weeks		After 24 weeks	
	I _{a,b,c,d,e}	II _{a,b,c,d,e}	III _{a,b,c,d,e}	IV _{a,b,c,d,e}	II _{b,c,e}	III _{b,c,e}	II _{b,c,e}	II _{b,c,e}
<i>Bacillus subtilis</i> NCTC 8236	++++	++++	++++	++++	+++	+++	+++	+++
<i>Bacillus pumilus</i> NCTC 8241	++++	++++	++++	++++	+++	+++	+++	+++
<i>Micrococcus luteus</i> ATCC 9341	++++	++++	++++	++++	+++	+++	+++	+++
<i>Staphylococcus aureus</i> NCTC 7447	++++	++++	++++	++++	+++	+++	+++	+++
<i>Escherichia coli</i> BPP01	++++	++++	++++	++++	+++	+++	+++	+++
<i>Pseudomonas aeruginosa</i> ATCC 10145	++++	++++	++++	++++	+++	+++	+++	+++
<i>Klebsiella pneumoniae</i> NCIB 9111	++++	++++	++++	++++	+++	+++	+++	+++
<i>Candida albicans</i> IMRU 3669	—	—	—	—	—	—	—	—
<i>Saccharomyces cerevisiae</i>	—	—	—	—	—	—	—	—
<i>Aspergillus fumigatus</i>	++++	—	—	++++	—	—	—	—
<i>Aspergillus niger</i>	—	—	—	—	—	—	—	—
<i>Aspergillus terreus</i> F	++++	++++	++++	++++	+++	++	+++	+++
<i>Penicillium citricus</i>	++++	++++	++++	++++	+++	+++	+++	+++
<i>Penicillium citricus</i> F	++++	++++	++++	++++	+++	+++	+++	+++

+ = inhibition

— = no inhibition

± = doubtful

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THE SEROLOGICAL SIGNS OF THE EPSTEIN–BARR VIRUS (EBV) ACTIVITY IN THE ELDERLY

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The authors determined the IgM and IgG anti-Epstein–Barr virus (EBV)-VCA and anti-EBNA levels in single serum samples of 1882 patients with diagnose except infectious mononucleosis. The serological results were divided into four groups according to different serological patterns: the seronegative group, the IgM anti-VCA positive group (supposedly having actual EBV infection), the IgG (only) anti-VCA group (probably recent EBV infection or not sufficiently functioning cellular immunity) and IgG anti-VCA antibodies together with anti-EBNA (past EBV infection). The presence of heterophil antibodies was detected too. The incidence rates of the serological patterns were classified according to different age groups. It has been observed that the EBV seropositivity rapidly increases with age and over 60 years of age it is over 96%.

At the same time the incidence rate of the IgM anti-VCA has gradually increased with age and from 3.66% in the age group of 0–10 years reached 11.98% in patients over 60 years of age. Significantly higher titres of IgG anti-VCA were found in the elderly group of patients as well. The distribution of heterophil antibody positive samples failed to show any significant distribution.

In 1964, Epstein, Achong and Barr reposed a unique new virus isolated from Burkitt's lymphoma [1]. Subsequently, EBV was shown to be associated with a variety of diseases, the most important being infectious mononucleosis (IM) [2–5]. Its role has been suspected in nasopharyngeal carcinoma [6–9] in immunocompromised conditions [10–14], Hodgkin's disease [15–20] and non-Hodgkin's lymphomas developing after bone marrow and solid organ transplantation [21–25]. The growing awareness of the importance of EBV has resulted in a vast amount of literature during the past ten years.

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The EBV itself is one of the eight known Herpes viruses which is ubiquitous and will have infected over 80% of individuals by the time they are adults [5, 28]. EBV contains a double-stranded molecule of DNA which is approximately 172 kb in length. Two types of EBV have been isolated worldwide, Type A and Type B. The incorporation of the EBV DNA genome into the host results in immortalization of peripheral blood B-cells to form lymphoblastoid cell lines [27–31].

Infection of the oral pharynx epithelium results in continuous shedding of infected virions in the saliva during a productive infection. The initial infection of EBV is thought to occur via aerosolized particles or through mouth-to-mouth contact [32, 33]. The epithelial cells in the oral and nasal pharynx contain a surface receptor for EBV. This receptor is similar to the CD3 component of complement and interferon [34, 35]. Incorporation of EBV DNA into B-cells results in a lifelong latent infection.

The primary EBV infection in children is most often asymptomatic. The primary infection in adolescents and young adults, however, usually results in the clinical symptoms associated with infectious mononucleosis in most individuals. In the normal, non-immunosuppressed individual, the pattern of antibody production to EBV-encoded proteins is predictable. Primary EBV infection is defined by the presence of anti-viruscapsid (VCA) IgM associated with symptomatology. Anti-VCA IgG is produced concurrently at a much lower level which increases over time. Anti-VCA IgG persists during the lifetime of an immunocompetent person. Anti EA-D (early antigen-diffuse) IgG antibodies are present in approximately 80% of immunocompetent individuals. Anti-EA-R (early antigen-restricted) IgG are rarely detected in the acute phase of infectious mononucleosis, however, they may be present during the convalescent period following EBV infection. Anti-EBNA (Epstein-Barr nuclear antigen) IgG is not present during acute infection, but it may be present during convalescence. A past infection is usually defined by the presence of IgG antibodies directed against VCA and EBNA in the absence of anti-VCA IgM. Approximately 90% of adults and only 20% of children are heterophil antibody positive. In cases where IM or other EBV driven processes are suspected, the determination of EBV specific antibodies are warranted.

Seroepidemiological surveys demonstrated that 90–97% of adults more than 60 years old were seropositive for EBV. The incidence of primary EBV infection manifesting as IM is particularly rare over 60 years of age and in the reported cases had significantly fewer occurrences of pharyngitis, lymphadenopathy [36].

In our present study we wished to determine the incidence of the primary EBV infection in different age groups by utilizing large numbers of serum samples from patients admitted with various diseases (except IM).

Materials and methods

Single serum samples were collected from 1882 patients admitted to the Department of Infectology, County Hospital, Szekszárd between January 1, 1984 and December 31, 1994 (11 years) with various diseases except infectious mononucleosis. Anti-EBV-VCA IgM and IgG were detected together with anti-EBNA and heterophil antibodies from each samples. The presence of antibodies to

EBV antigens was determined by indirect immunofluorescence techniques published in the literature [5, 32, 37].

B95-8 EBV transformed monkey lymphoblastoid cells maintained in Eagle MEM medium containing 10% calf sera were used to detect anti-EBV-VCA IgM and IgG. Drops of cell suspension expressing different EBV antigens were spread on glass slides and fixed in acetone were used to detect EBV antibodies. Serum dilutions of 1:5–1:1280 were used to detect anti-VCA IgG and sera dilutions of 1:5–1:320 to detect anti-VCA IgM. The slides were incubated for 60 min and repeatedly washed in PBS (Dulbecco's Phosphate Buffered Saline, pH 7.2–7.4) then covered with FITC-conjugated antihuman IgG and IgM (DAKO) dilutions. After repeated washes, Evans blue contrast stain was applied. The cells were covered with glycerol-PBS and examined under the fluorescence microscope. The result was considered as positive if the given serum dilution markedly produced VCA staining. In the case of anti-EBV-VCA IgM detection the presence of rheuma factor was determined, too.

Suspension of Raji cells was used to detect EBNA antibody by anticomplement immunofluorescent (ACIF) method utilizing EBV antibody-free human sera as complement source and anti-C36-FITC (DAKO). The reaction was marked as "positive" when a characteristic fluorescence in the nucleus was seen.

Paul-Bunnell-Davidson test was used to determine heterophil antibody simultaneously from each sample.

The results, depending on the serological patterns, were divided into four groups: 1. Seronegative group; 2. Anti-EBV-VCA IgM positive group suspected of having actual EBV infection; 3. Anti-EBV-VCA IgG positive only group (without EBNA or IgM) suspected of recent EBV infection (or reduced cellular immunity); 4. Anti-EBV-VCA IgG positive group together with anti-EBNA simultaneously.

The patients were divided into five age groups: 0–10, 11–20, 21–40, 41–60 and over 60 years of age.

The incidence rates of serological patterns in the different age groups were estimated and expressed in per cent.

The anti-VCA IgG titres were divided in "low" (1:10–1:80), "medium" (1:160, 1:320) and "high" (1:640 and above) titre groups and their incidence was determined in the five age groups. The incidence rates were compared and significance calculated by Chi-square probe

Results

The distribution of the serological patterns in different age groups is demonstrated in Table I. It can be observed that the rate of seronegativity rapidly decreases in the higher age groups and over sixty years of age it is under 4% (3.42%). The incidence rate of anti-EBV-VCA IgM, which is only 3.66% among the patients of 0–10 years of age, starts to increase from the 21–40 years age group and in patients over 61 years is nearly 12%. The serogroup of patients with anti-EBV-VCA IgG only failed to show any significant variation among different age groups, but remained constantly at the same level (25–26%), while the rate of anti-EBNA containing samples have reached 60% over 10 years of age and remained practically at the same level in every age group.

The distribution of anti-EBV-VCA IgG titres ($n=1662$) among different age groups showed that the rate of "low" titres of IgG (1:10–1:80) ranged between 33.68% in the group of patients over 60 years and 48.07% among patients between 11–20 years of age. The difference is not significant. No significant change was observed among different age

groups in the case of "medium" titres (1:160–1:320), but the samples with "high" titres of IgG anti EBV-VCA (1:640 and above) showed a steady increase in age groups and its rate doubled from the younger age group to the oldest one (11.45%–23.04%). The difference is significant ($P<0.01$).

When the group of patients with anti-VCA IgG only was analysed ($n=491$) in the same way, this kind of distribution pattern was much more obvious. The rate of "low" titre of IgG anti-VCA dropped from 60.55% in the group of patients of 11–20 years of age to 38.96% over 60 years. This difference is significant ($P<0.01$). The rate of "high" titre of IgG of anti-VCA increased from 8.62% in the youngest age group to 27.27% in the patients over 60 years of age and this difference was significant also ($P<0.01$) (see Figs 1, 2).

Table I

Distribution of different serological patterns between age groups in % ($n=1882$)

Age in years	Seronegative		IgM anti-VCA positive		IgG (only) anti-VCA positive		IgG anti-VCA + anti-EBNA pos.	
	n	%	n	%	n	%	n	%
0–10 years	149	34.1	16	3.66	116	26.54	156	35.69
11–20 years	22	9.56	8	3.47	61	26.52	139	60.43
21–40 years	21	4.04	28	5.39	131	25.21	339	65.31
41–60 years	18	4.45	36	8.91	106	26.23	244	60.39
over 60 years	10	3.42	35	11.98	77	26.36	170	58.21

Heterophil antibodies were found in 42 samples, out of them 12 samples were positive for IgM anti-VCA, too. No significant distribution among age groups were observed.

Discussion

Primary EBV infection occurs in the majority of individuals during childhood, when infection is asymptomatic, or during adolescence, when up to 60% of those who acquire the virus have clinically overt disease. Infection with this virus has ramifications for the elderly population as well. Little is known about the consequences of persistent EBV infection in the elderly host. Seroepidemiologic studies of EBV show a high prevalence in different countries of IgG anti-VCA by age 30. Our results indicated, that EBV seropositivity is over 95% in the population older than 60 years of age.

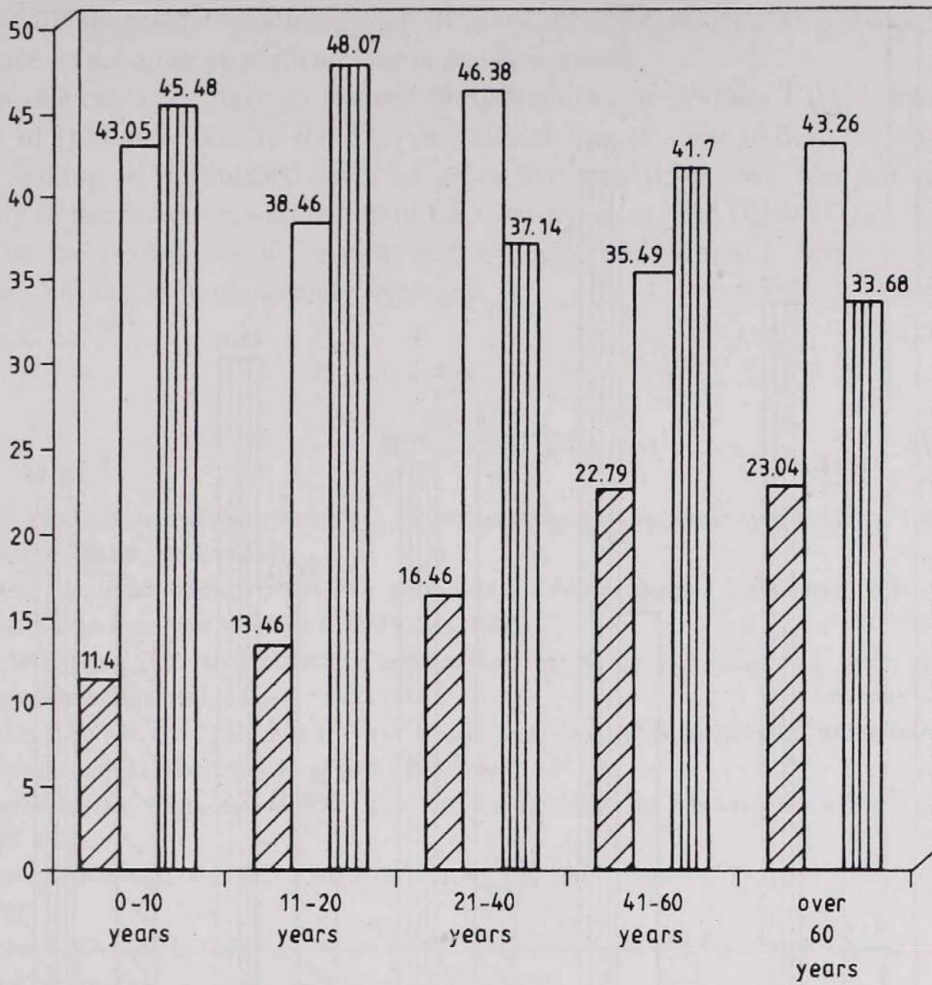


Fig. 1. Distribution of IgG anti-VCA titres in different age groups in % (n=1662). Shaded columns: high (1:640 or over); solid columns: medium (1:160-1:320); clear columns: low (1:10-1:80). The distribution of different titres of serum samples positive for anti-EBV VCA IgG shows a tendency of increasing incidence of the samples with "high" titres among elderly patients. (The rate has increased from 11.4% to 23.04%)

The gradually increasing incidence rate of IgM anti-VCA seropositivity, which is 11.98% among patients over 60 years of age calls the attention to the possibility of an actual EBV infection (reinfection, reactivation). This possibility is supported further by the observation that high titres of IgG anti-VCA are found significantly more frequently among elderly patients.

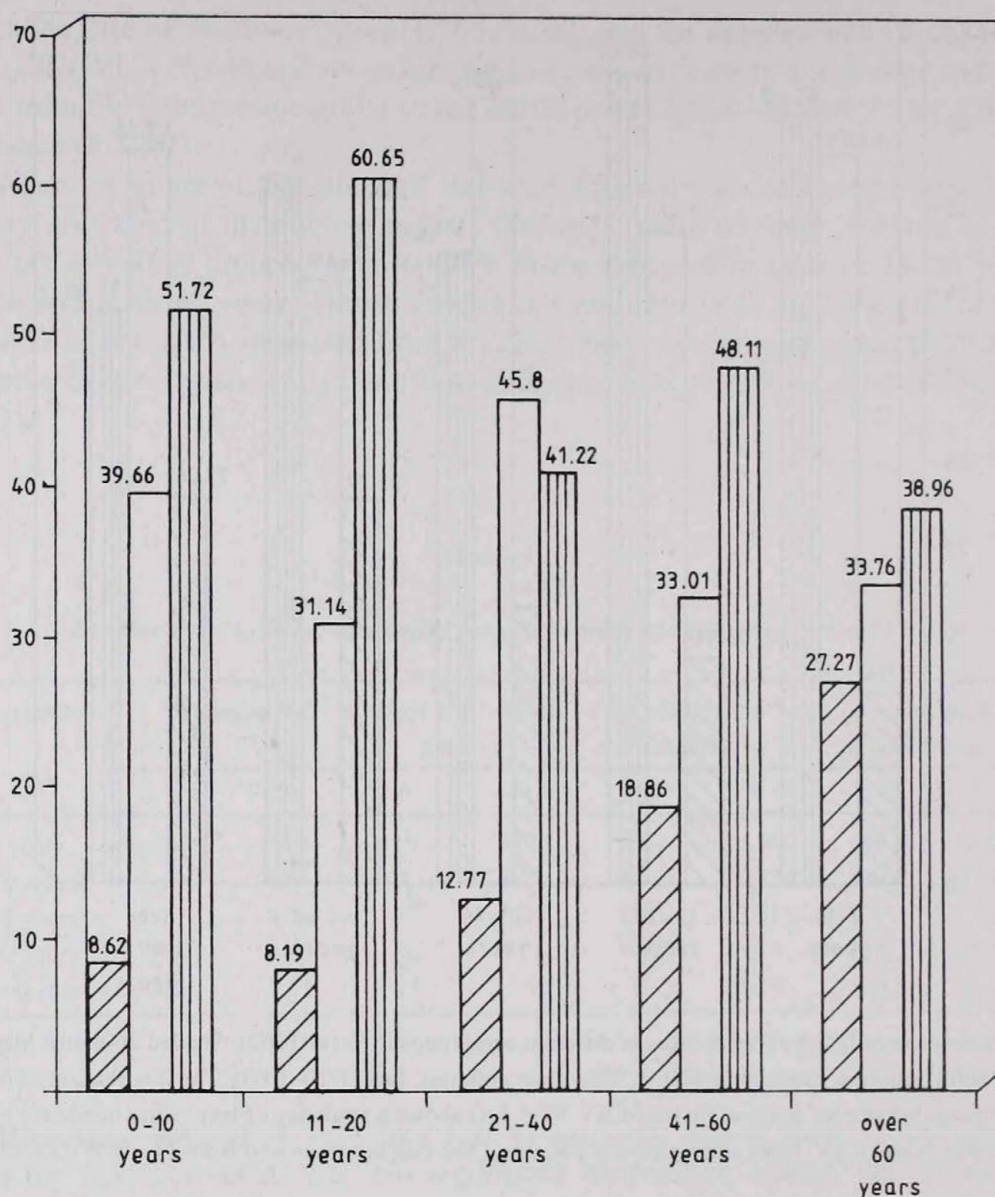


Fig. 2. Distribution of IgG (only) anti-VCA titres in different age groups in % (n=491). Shaded columns: high (1:640 or over); solid columns: medium (1:160–1:320); clear columns: low (1:10–1:80). Distribution of different titres of serum samples positive for anti-EBV VCA IgG only shows a tendency of increasing incidence of the samples with “high” titres among elderly patients. (The rate has increased from 8.62% to 27.27%)

Schmader et al. observed elevated antibodies to EBV among healthy elderly adults [37]. Sumaya et al. [35] found that elderly subjects had the highest geometric mean titres of IgG to VCA among adults. In the cases of permanent seronegativity, the lack of receptors on B cells can be suspected [18].

The actual frequency of active EBV replication and excretion in the elderly is unknown. Clinical manifestations of EBV infection in the elderly included typical and

atypical mononucleosis associated with primary infection as well as possible neoplastic events such as nasopharyngeal carcinoma and lymphoma.

In our cases we have to suspect the possibility of primary EBV infection in the presence of IgM anti-VCA or the EBV reinfection due to some underlying conditions or old age leading to diminished level of protective antibodies. We can not exclude the possibility of the infection with different EBV serotypes as well (EBV1-2).

The full expression of the virus in the elderly and its role in less obvious clinical syndromes has not been completely explored.

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SEROTYPING AND VIRULENCE FACTORS OF *PSEUDOMONAS AERUGINOSA* CLINICAL ISOLATES

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Pseudomonas aeruginosa is one of the major opportunistic human pathogens very often responsible for severe infections mainly in immunocompromised hosts. These bacteria may cause the broad spectrum of infections which are associated with urinary, respiratory and gastrointestinal tract, burn wounds, eyes as well as with other sites [1, 2]. *P. aeruginosa* also significantly contributes to high morbidity and mortality rates of nosocomial infections [3, 4]. This microorganism produces a number of exoproducts which have been implicated in the pathogenesis of pseudomonas infections [5–8]. Among them, the activity of elastase, proteinase and alginate were well established [9–12].

The aim of the present study was to determine the distribution of *P. aeruginosa* strains (isolated from urinary and respiratory tract infections) in O-serotypes and to evaluate some of their characteristics (elastase, proteinase and alginate).

The distribution of 53 *Pseudomonas aeruginosa* strains (20 urinary, 33 respiratory) in O-serotypes as well as determination of their three virulence factors were evaluated. Serotype O11 was the most frequent in both of groups (24.5% respiratory isolates, 18.9% urinary ones). Urinary as well as respiratory strains showed elastase (90% or 84.9%), proteinase (85% or 93.9%) and alginate (85% or 90.9%). Lower level of elastase (1.0–4.9 µg/ml) and alginate production (1.0–49.9 µg/ml) was manifested by the majority of urinary (80% or 55%) as well as respiratory isolates (51.5% or 57.6%). On the other hand, 60% of urinary and 63.6% of respiratory strains produced proteinase at a higher level (10.0–20.0 µg/ml). Most strains belonging to the predominant serotype O11 showed lower elastase, but higher level of proteinase and alginate levels.

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Material and methods

Bacterial strains. Clinical *P. aeruginosa* strains (53) were isolated from different urinary (20) and respiratory tract infections (33). The strains were subjected to a number of test-tube biochemical tests (13).

Serotyping of strains was done by slide agglutination method with live antigens from an overnight (20–24 h) blood agar culture using agglutinating antisera (4 mixtures of sera and 16 monovalent ones; Diagnostics Pasteur).

Hemolytic activity was followed up after incubation of bacteria (24 h, at 37 °C) on agar containing 6% of sheep blood.

Preparation of culture filtrates. Aliquots of 0.6 ml of bacterial suspension ($A_{600}=0.5$) were inoculated into 29.4 ml of Mueller–Hinton broth (supplemented with 25 mg of CaCl_2 and 12.5 mg of MgSO_4 per litre) and incubated on a reciprocal shaker at 37 °C for 24 h. Sterile culture filtrates for determination of enzymatic activity and alginate were obtained by centrifugation of bacterial suspensions and filtration of their supernatants (0.22 μm , Millipore).

Elastase activity was determined spectrophotometrically at 495 nm [14]. Elastin Congo red was used as substrate. The values of elastase (mg/l) were determined from the standard curve prepared with elastase type IV (Serva). Values less than one were considered negative. Because numerical values of *P. aeruginosa* virulence factors were heterogeneous their means were not determined and exoproducts were classified to the groups with certain range of values.

Proteinase activity was evaluated spectrophotometrically at 260 nm [15]. Casein (Hammarsten, Serva) was used as substrate. The proteinase values (mg/l) were read from the standard curve with bacterial protease, type XXIV (Sigma).

Alginate determination was carried out spectrophotometrically at 530 nm by borate carbazole assay at 55 °C [16, 17]. The alginate amount (mg/L) was read from the standard curve prepared using mannuronolactane (Sigma).

Results

The distribution of all strains tested in O-serotypes is displayed in Table I. Serotype O11 was the most frequent in both the groups (24.5% respiratory isolates, 18.9% urinary ones), followed by serotypes O4, O6, O10 (each 7.5%) in respiratory strains and by polyagglutinating strains (5.7%) as well as by serotypes O4 and O6 (each 3.8%) in urinary strains. All strains tested were haemolytic on agar plates containing 6% sheep blood.

Our further results showed that most strains tested produced elastase (90% urinary and 84.9% respiratory) (Table II). The amount of this enzyme varied. The majority of urinary isolates (80%) as well as respiratory strains (51.5%) produced elastase in the range of 1.0–4.9 $\mu\text{g/ml}$. On the other hand smaller part of the strains (10% urinary or 33.4% respiratory) showed higher elastase level (5.0–10.0 $\mu\text{g/ml}$).

Also proteinase was produced by most of the strains (85% urinary or 93.9% respiratory). Approximately the same percentage of urinary strains (25%) as well as respiratory ones (30.3%) showed lower proteinase level (1–9.9 $\mu\text{g/ml}$) in comparison with the majority of the strains both of groups (60% urinary or 63.6% respiratory) which

produced this enzyme in higher extent (10.0–20.0 µg/ml). Eighty-five per cent of urinary strains as well as 90.9% of respiratory isolates produced alginate (Table II). More than 50% of urinary strains (55%) or respiratory strains (57.6%) manifested lower alginate level (1.0–49.9 µg/ml) in comparison with 30% of urinary strains and 33.3% of respiratory ones which produced larger amounts of alginate (50.0–100 µg/ml).

When looking for a relationship between all the strains tested, belonging to the predominant serotype O11 and their virulence factors, we observed that most of the strains (78.3%) produced elastase at lower level (1.0–4.9 µg/ml), on the other hand 73.9% or 52.2% of these isolates showed higher proteinase (10.0–20.0 µg/ml) as well as alginate level (50.0–100 µg/ml) (Table III).

Table I

Distribution of P. aeruginosa strains according to O-serotypes (O1–O16)^a

Source of strains	O1	O2	O4	O6	O7	O9	O10	O11	O13	PA ^b	NT ^c
Urinary No.	0	0	2	2	1	1	1	10	0	3	0
%	0	0	3.8	3.8	1.9	1.9	1.9	18.9	0	5.7	0
Respiratory No.	1	2	4	4	0	0	4	13	2	2	1
%	1.9	3.8	7.5	7.5	0	0	7.5	24.5	3.8	3.8	1.9
Total No.	1	2	6	6	1	1	5	23	2	5	1
%	1.9	3.8	11.3	11.3	1.9	1.9	9.4	43.4	3.8	9.4	1.9

^aSerotypes O3, O5, O8, O12, O14, O15 and O16 were not detected

^bPA = polyagglutinating strains

^cNT = nontypable strain

Discussion

Our results showed that all *P. aeruginosa* strains tested were distributed in three predominant O-serotypes (O11, O4, O6) followed by serotype O10 and by a group of polyagglutinating strains. These results are comparable with those of other authors showing the prevalence of the same serotypes [18–20]. Serotype O12 was not encountered among our strains. A low incidence of serotype O12 (2.3%) was found by Bert and Lambert-Zechovsky [21]. It is known that serotype O12 (widely occurring mainly in Europe) is often involved in nosocomial infections.

Table II

Evaluation of P. aeruginosa virulence factors

Urinary tract				
(% of P ^a)	P ^a		P ^a	(% of P ^a)
		Elastase (µg/ml)		
(80)	16	1.0–4.9	17	(51.5)
(10)	2	5.0–10.0	11	(33.4)
		Proteinase µg/ml		
(25)	5	1.0–9.9	10	(30.3)
(60)	12	10.0–20.0	21	(63.6)
		Alginate µg/ml		
(30)	6	1.0–24.9	10	(30.3)
(25)	5	25.0–49.9	9	(27.3)
(10)	2	50.0–74.9	6	(1)
(20)	4	75.0–100	5	(15.1)

P^a = Number of positive strains

Table III

Levels of virulence factors^a of P. aeruginosa strains belonging to serotype O11

Virulence factors µg/ml	Strains	
	No.	%
Elastase		
1.0–4.9	18	(78.3)
5.0–10.0	3	(13.0)
Proteinase		
1.0–9.9	5	(21.7)
10.0–20.0	17	(73.9)
Alginate		
1.0–24.9	5	(21.7)
25.0–49.9	4	(17.4)
50.0–74.9	8	(34.8)
75.0–100	4	(17.4)

^aVirulence factors; two strains belonging to serotype O11 did not produce elastase and alginate, one strain proteinase

On the other hand published data showed that also other *P. aeruginosa* serotypes were predominant. Puzová et al. [22] found that 42.4% of urinary strains were nontypable, similarly Zatkovič et al. [23] observed the most frequent nontypable strains were isolated from urine and sputum.

The majority of the strains tested produced elastase, proteinase and alginate. Hamood et al. [24] likewise observed that most *P. aeruginosa* isolates (tracheal, urinary tract and wound infections) manifested some extracellular virulence factors, including elastase.

In our study more than 50% of urinary as well as respiratory strains showed lower production of elastase and alginate, on the other hand higher levels of proteinase were found. Literary data concerning the production of *P. aeruginosa* exoproducts are different. Large amount of proteinase but also of elastase production by urinary strains was reported by Woods et al. [25], Janda and Bottone [26] found low levels of elastase manifested by urinary strains.

The majority of the strains tested belonging to the predominant serotype O11 produced lower elastase levels but higher amounts of proteinase and alginate. High levels of total protease but also of elastase production by strains belonging to serotype O11 was found by Vicsa et al. [20].

Literary data indicated that determination of virulence factors production by *P. aeruginosa* strains can be important because of the connection between higher levels of some exoproducts and type of infections is suggested [24].

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CONTRIBUTION TO THE STUDY OF SURFACE MICROFLORA ON CANTAL CHEESE

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Bacteria and mould were isolated from microflora of Cantal cheese rind. They were identified and the influence of pH, temperature and water activity on growth rate were studied. An equation for growth rate $\mu(h^{-1})$ relative to temperature is described according to a mathematical model.

Cantal is a semi-hard pressed cheese made with raw milk. It is an "Appellation d'Origine Contrôlée" cheese with production limited to the French department of Cantal and some townships in Aveyron, Corrèze, Haute-Loire and Puy de Dôme.

Rind formation is one of the most important phases in the Cantal manufacturing process. The cheese-maker always attempts to obtain a thick rind with bulges and craters penetrating into the golden to orange-yellow cheese appearance. Cheese-makers call this the "Boutonné". The influence of physicochemical parameters of Cantal cheese on the growth of isolated microorganisms was studied.

Materials and methods

Study of bacteria. All cultivations were carried out in Erlenmeyer flasks filled to one tenth of their volume and shaken (80 oscillations per min; amplitude 7 cm). They were incubated at 28 °C except where otherwise indicated. The basal medium was TYG (tryptone 0.6% w/v, yeast extract 0.3% w/v and glucose 0.5% w/v); [1]. This medium was sterilized by autoclaving at 120 °C for 20 min.

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Physicochemical parameters (pH and temperature) were studied as previously described [2]. For control water activity (A_w), NaCl and sorbitol were kept at concentrations determined from values reported by Bizot [3].

Nepierian growth rate determinations. Growth was studied by monitoring the optical density at 500 nm. Nepieran growth rates ($\mu_{\text{h}^{-1}}$) were determined from growth curves [4].

Growth modeling. Mathematical models of growth rate as a function of temperature were used as described by Vivier et al. [5].

Identification tests. All isolated bacteria were determined according to Baird-Parker [6] and Schneifer et al. [7].

Study of Moniliella sp. Growth studies were carried out on DYAA (Dextrose Yeast Asparagine Agar), as described in the MUCL Catalogue [8]. They were incubated at 18 °C, except for the special conditions. The basal medium was sterilized by autoclaving at 120 °C for 20 min.

The influence of physicochemical parameters (pH, temperature, A_w) were studied according to Ratomahenina et al. [9].

Growth of mould was estimated by measuring the increase in colony diameters. An average of 15 determinations gave the radial growth rate. The results are expressed as the percentage of maximal growth rate.

The *Moniliella* strain isolated in the study was inoculated in industrial manufacturing conditions during curd milling and at the surface of loaves by spreading an aqueous spore solution of the mould.

Results and discussion

The three main types of bacteria were identified, according the method of Schneifer et al. [7] for species determinations, as *Micrococcus halobius* (a white bacterium), *Micrococcus sedentarius* (a yellow bacterium) and *Micrococcus roseus* (an orange bacterium).

The optimum pH for growth was about 8 for *M. halobius* and *M. sedentarius*. Growth was not observed at pH 3 and pH 4, and both bacteria grew poorly between pH 5 and pH 7. For *M. roseus*, the optimal pH was about pH 9 and the minimum pH for growth was 4. The pH of the cheese rind was about 7.5 (Fig. 1).

The results are given in Fig. 2. Our experimental conditions (liquid medium) were quite different from conditions on the surface of the cheese. In the presence of NaCl, *M. halobius* (Fig. 2A) grew suitably to 10% NaCl with $\mu=0.3 \text{ h}^{-1}$. At 20% NaCl, growth was not observed. Growth of *M. sedentarius* (Fig. 2B) and *M. roseus* (Fig. 2C) was strongly inhibited at 5% NaCl. Indeed, when 10% NaCl was used, the Nepieran growth rate was only 0.04 h^{-1} for *M. sedentarius* and 0.09 h^{-1} for *M. roseus*. At higher NaCl concentrations, growth was not observed. The NaCl content in the rind was around 1.2% w/w.

The results obtained with NaCl and sorbitol were comparable. The action of NaCl could be explained by its ability to suppress water activity.

Figures 3A, 3B and 3C depict growth rate as a function of temperature (K) for *M. halobius*, *M. sedentarius* and *M. roseus*, respectively. The optimal and minimal temperatures were calculated from the experimental results using the Ratkowski equation [10] and found to be about 8 °C and 32 °C, respectively, for the three cultures.

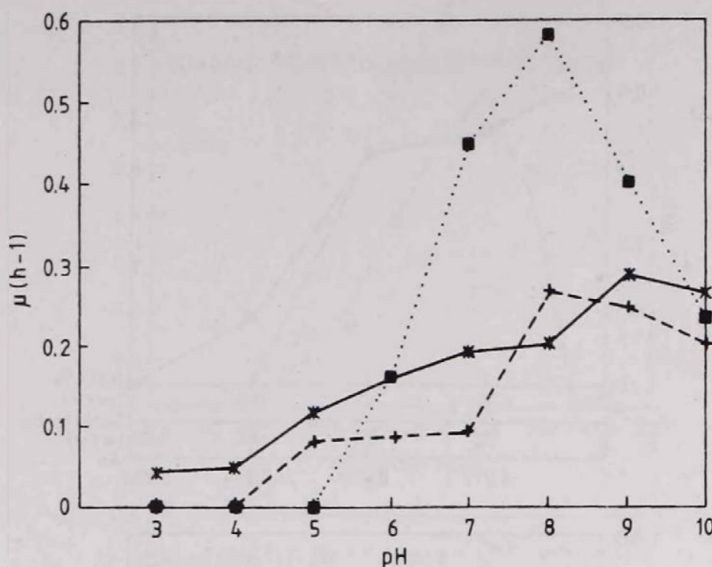


Fig. 1. Effect of pH on the growth of ■ *M. halobius*, + *M. sedentarius*, * *M. roseus*

The minimum temperature ($T_{\min}$) represents an intrinsic property of an organism when growth conditions other than temperature are non-limiting [10].

The isolated mould was identified as a *Moniliella* species. It was a white velvety mould that became pale pink-orange after a few days.

The results are given in Fig. 4A. Growth rates were determined at 18 °C on DYAA medium buffered to different extents. This mould was able to grow suitably at a pH range of 6 to 10.

The results are given in Fig. 4B. Growth rates are expressed as a function of sodium chloride contents (5 to 20% w/v). It was found that at water activity values of up to 0.942 (corresponding to 10% NaCl), growth was weakly inhibited. Up to this value, growth decreased and was nil at 20% NaCl. Sorbitol gave similar results.

As noted in Fig. 4C, the optimum temperature for growth was found to be about 18 °C. At 10 °C, which is the temperature of cellars where ripening is carried out, growth was about 70% of the maximal rate. At 4 °C, growth was weak (15% of the maximal rate).

The surface flora of Cantal cheese is very complex. *M. roseus* and *M. sedentarius* certainly play a role in obtaining the desired outer appearance of Cantal. Other bacteria such as *Brevibacterium linens* may also be involved. *M. halobius* clearly grows well in cheese-making conditions and probably interferes with the proliferation of other bacteria which contribute to the colour of the cheese.

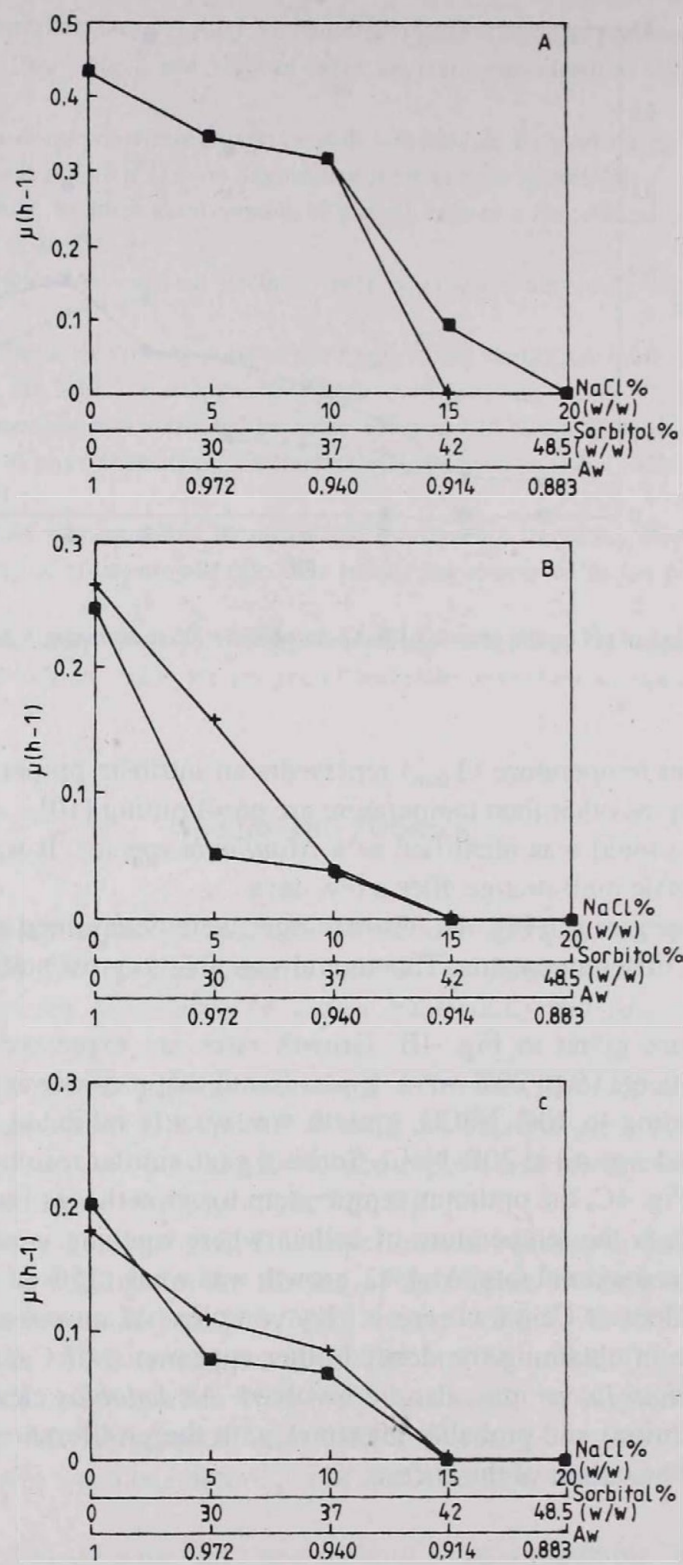


Fig. 2. Effect of NaCl and water activity on the growth of A: *M. halobius*, B: *M. sedentarius*, C: *M. roseus*.
■ NaCl, + sorbitol

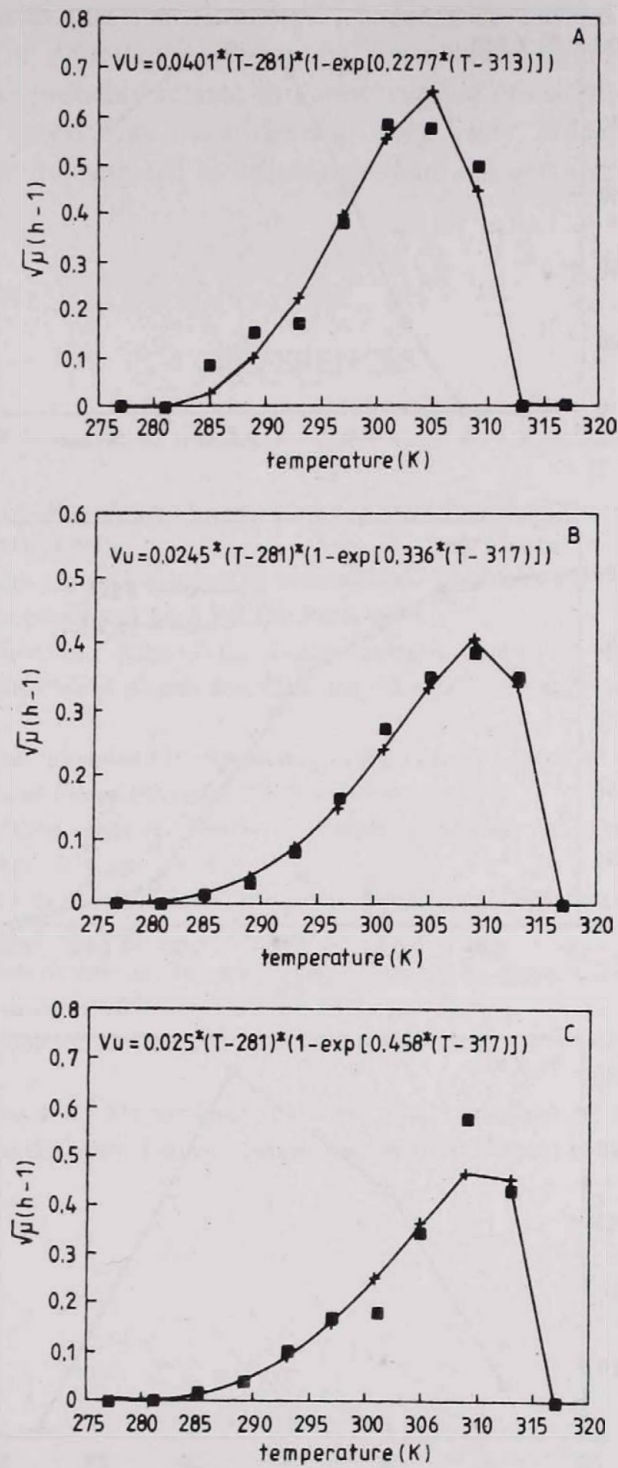


Fig. 3. Modeled effect of temperature on growth. A: *M. halobius*, B: *M. sedentarius*, C: *M. roseus*.
 ■ Experimental values, + theoretical values

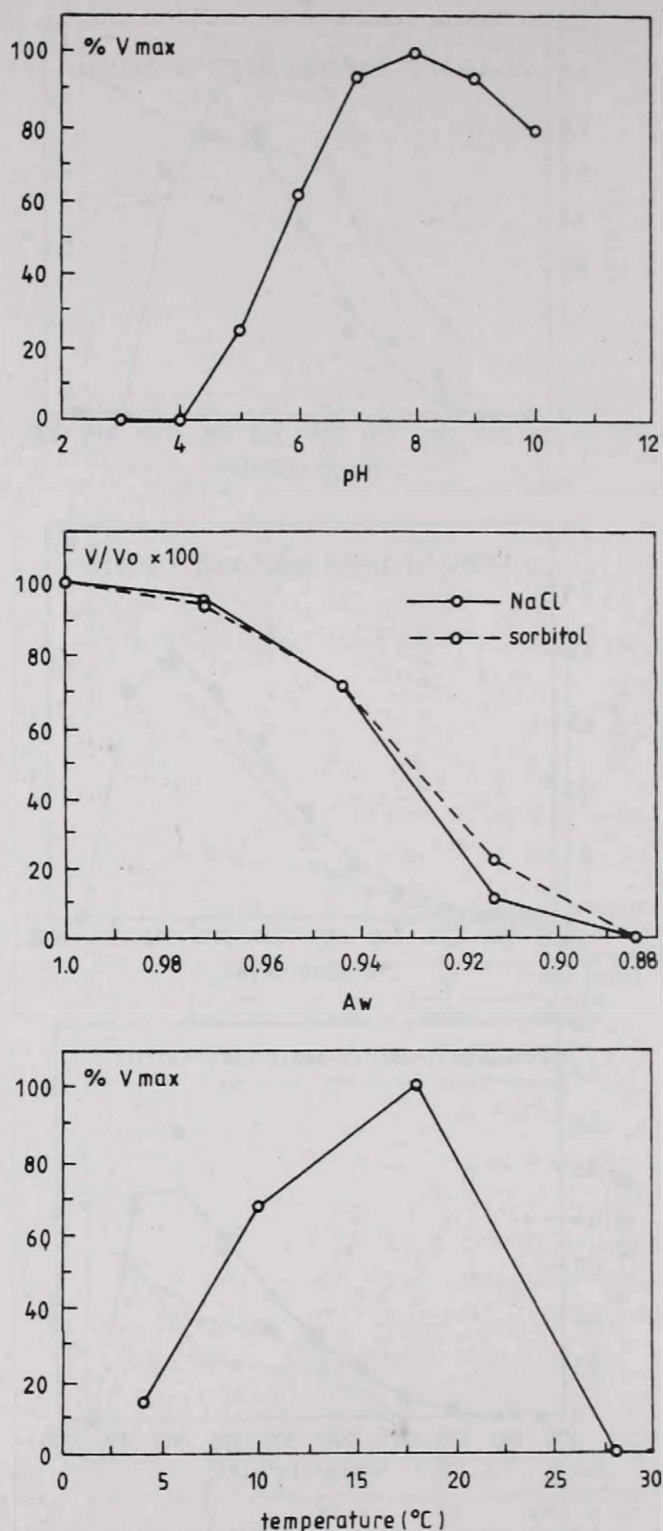


Fig. 4. Effect of pH (A), water activity (B) and temperature (C) on growth of *Moniliella* species.
 V: growth rate with NaCl o— or sorbitol o-----; Vo: growth rate without NaCl and without sorbitol;
 Vmax: optimal growth rate

It is well known that the "Boutonné" appearance desired by cheese-makers is related to colonies of *Moniliella* sp., among other microorganisms. In addition, the "Boutonné" feature is probably related to the successive development of different flora. *M. roseus* and *M. sedentarius* must develop very early. The kinetics of microbial interactions should be investigated to fully understand the occurrence of the "Boutonné" feature.

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CHANGES IN WATER ACTIVITY AND SOME MICROBIAL GROUPS DURING STORAGE OF PET FEED

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The effect of exposure of different pet feed samples to the typical temperature and moisture conditions of many months of the year (9 °C and 80% RH) on water activity (a_w), pH and growth of various microbial groups (mesophilic aerobic bacteria, fungi and yeasts) was studied. The storage conditions were found to scarcely influence the samples pH, with no significant changes relative to the initial values (the standard deviation was 0.04 and the coefficient of variation 0.85%). By effect of exposure to the temperature and moisture conditions tested, the initially stable feeds reached moisture contents close to 20% and a_w values near those of equilibrium with the ambient relative humidity during storage. Microbial counts increased with increasing a_w , with final means of 8.44, 9.16 and 8.71 log CFU/g for mesophilic aerobic bacteria, fungi and yeasts, respectively. The risk of mycotoxin production at these a_w values is discussed. Careful control of moisture is recommended in order to preserve the quality of pet feed throughout its scheduled shelf life.

The moisture content and water activity (a_w) are two critical parameters that influence the storage life of most foods [1, 2]. In many cases, the presence of excessive amounts of water can lead to deterioration; in others, water losses can render foods unusable. Proper control of the amount of water in foods therefore requires a precise knowledge of their critical moisture content, as well as the ambient temperature and moisture conditions during storage.

The shelf life of foods spans the period during which it can be used by consumers or expected to preserve its quality. The criteria usually employed to assess shelf life rely on quality measurements based on microbial decay, changes in colour, texture and nutrient contents, and lipid oxidation. Additional subjective factors such as flavour, texture and colour can be used as reliable indicators for predicting the shelf life of a given food [3]. The main objective of both traditional and innovative food preservation processes is to inhibit microbial growth. Thus, one of the more common procedures for making pet food involves the preparation of feed containing a wide variety of ingredients the stability of which against microbial alteration is ensured by lowering the water

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activity, a_w , below 0.60. This type of feed is quite stable and is only altered by oxidative rancidification or exposure to a highly moist atmosphere, which can lead to rehydration and to increased a_w values and microorganism growth.

Because some pet feeds are sold in bulk in open bags the contents of which may be exposed for long periods to the shop's ambient conditions, the aim of this work was to study the effect of exposure of various feed samples on water activity, pH and some microbial groups (the factors that determine conservability in this type of product).

Material and methods

Samples. An overall 20 samples of pet feed [4, 5] were purchased at supermarkets and specialist shops. Eight of the samples (nos 1–8) were dog feed and the other twelve (nos 9–20) cat feed. The samples, in various marketing sizes (400 g and 1.5, 3 and 4 kg), were chosen from the most commonplace brands.

Storage conditions. Following transfer to the laboratory, the samples (400 g and 1.5 kg cardboard boxes, and 4 kg paper bags) were opened and stored in a B-3-400 weather chamber from ASL Aparatos Científicos (Madrid, Spain) at 9 °C and 80% RH. Analyses were carried out after 0, 5, 10, 15 and 20 days of storage.

The moisture gain during storage was determined by placing the samples in large dishes and weighing them after the above-stated storage times in the weather chamber. Simultaneously, all other physico-chemical parameters were determined.

Physico-chemical analyses. Samples were prepared according to AOAC's recommended procedure [6]. Experimental determinations of a_w were conducted at 20 °C by using a CX-1 dew-point hygrometer from Decagon Devices (Pullman, WA) that was calibrated with saturated saline solutions [7]. pH values were obtained potentiometrically by means of a Beckman 3500 digital pH-meter applied to a 1:1 w/v aqueous homogenate. Finally, ash and total chloride were determined according to AOAC [6].

Microbiological analyses. For sample preparation and microbial counting, decimal dilutions were prepared from aseptically weighed samples of 10 g in a Stomacher bag that was supplied with 90 ml of a sterile solution of 0.1% peptone water. The mixture was homogenized in the bag for 2 min and subsequently used to prepare the following dilutions.

The above-mentioned media and conditions were used to perform the following counts: (a) Mesophilic aerobic bacteria [8], with plate count agar (PCA, Oxoid CM 325), following incubation at 30 °C for 72 h; (b) Fungi and yeasts [9], with potato dextrose agar (PDA, Oxoid CM139) acidified to pH 3.5 with 10% tartaric acid, after incubation at 26 °C for 96 h. Counts were obtained by morphological identification of typical colonies. All counts are given as log CFU/g, where "CFU" denotes colony forming units.

Results

Table I gives the water activity (a_w), pH and moisture, ash and NaCl contents in the samples prior to storage. The mean water activity values obtained fall within the range for low moisture foods ($0.00 < a_w < 0.60$) with the exception of samples no. 13 and no. 15, which were slightly above the upper limit (0.623 and 0.633, respectively).

Table I

*Water activity (a_w), pH, moisture, ash and NaCl contents in pet feed**

Product	Sample no.	a_w	pH	Moisture	Ash	NaCl
				g/100 g		
Ox croquettes	1-2	0.385-0.574	5.43-5.43	6.78-9.57	6.34-6.82	1.06-0.87
Chicken and rice croquettes	3-4	0.507-0.508	5.61-5.60	7.19-7.22	6.63-6.66	0.94-0.92
Growing formula croquettes	5-6	0.392-0.383	5.53-5.54	6.49-6.63	7.12-6.99	1.18-1.21
Chicken and cereal croquettes	7-8	0.435-0.377	5.19-5.75	6.05-6.01	7.41-6.60	0.99-1.00
Meat croquettes	9-10	0.401-0.405	4.88-5.39	7.18-7.68	8.15-7.59	2.65-2.25
Ox, veal and vegetable croquettes	11-12	0.492-0.527	4.92-5.06	7.17-7.83	6.83-7.17	0.88-0.91
Meat and fish croquettes	13-14	0.623-0.511	4.75-4.78	7.21-7.27	7.32-7.19	0.57-0.55
Trout, salmon and tuna croquettes	15-16	0.633-0.507	5.08-5.02	8.10-8.33	6.49-7.01	1.15-1.15
Tuna croquettes	17-18	0.353-0.389	4.85-4.62	5.09-6.61	8.17-8.07	0.27-0.23
Rabbit, liver and carrot croquettes	19-20	0.537-0.535	4.95-4.93	7.56-7.58	7.32-7.37	0.95-0.96

* Initial values

Table II

Changes in a_w and the moisture content in pet feed during storage at 9 °C and 80% RH

Product	Sample	Storage time (days)					Storage time (days)				
		0	5	10	15	20	0	5	10	15	20
		a_w					Moisture content				
Ox croquettes	1-2	0.480	0.733	0.759	0.789	0.788	8.18	11.26	14.93	14.80	15.57
Chicken and rice croquettes	3-4	0.508	0.650	0.724	0.753	0.785	7.22	8.72	10.05	20.17	20.53
Growing formula croquettes	5-6	0.388	0.704	0.738	0.749	0.787	6.56	8.79	13.19	12.97	15.03
Chicken and cereal croquettes	7-8	0.406	0.712	0.742	0.737	0.787	6.03	7.43	11.18	10.94	12.52
Meat croquettes	9-10	0.403	0.714	0.746	0.756	0.774	7.43	10.59	14.72	14.84	16.08
Ox, veal and vegetable croquettes	11-12	0.510	0.670	0.727	0.733	0.774	7.50	8.13	11.42	21.60	21.58
Meat and fish croquettes	13-14	0.567	0.699	0.724	0.764	0.787	7.24	8.79	11.27	20.98	21.05
Trout, salmon and tuna croquettes	15-16	0.570	0.700	0.718	0.758	0.781	8.22	9.28	12.43	22.64	22.38
Tuna croquettes	17-18	0.371	0.692	0.749	0.765	0.799	5.85	7.88	11.40	12.51	13.63
Rabbit, liver and carrot croquettes	19-20	0.537	0.669	0.733	0.773	0.796	7.56	9.00	12.23	22.18	23.16

pH values ranged from 4.62 and 5.75; most, however, were close to 5 or even higher. These pH values are low enough to inhibit microbial growth by themselves since major detrimental germs have a growth-limiting pH of 5–5.7 most moulds and yeasts have very low minimum pH values (3 and 2, respectively) [10].

The ash contents obtained, between 6.34 and 8.17%, were consistent with the percent compositions given on the product labels. The total chloride contents found are worth special note, however. As can be seen in Table I, such contents ranged from 0.23 to 2.65% and were close to 1% in most cases; therefore, they could hardly restrict microbial growth since inhibiting the development of species causing nutritional toxicity has been reported to require chloride contents above 6% [11–14] unless additional hindrances such as desiccation, temperature, the redox potential, thermal treatment or another additive such as sodium nitrite or polyethyleneglycol exist.

Table II shows the changes in a_w and the moisture content of the feed samples throughout the storage period. As can be seen, all the samples studied had a_w values within the range of intermediate moisture foods (IMFs) that increased gradually to 0.80 in virtual equilibrium with the ambient RH.

From the results in Table II it follows that moisture increased in parallel with a_w ; in fact, the initial values (6–8%) rose above 20% in some cases. Therefore, these initially stable products become IMFs and hence more prone to stability problems within a few days.

Despite their low moisture contents, IMFs undergo many detrimental chemical reactions. Their ease of oxidation and hence their shelf life depend on the initial composition, moisture content, partial oxygen pressure, amount of oxygen available in the package and permeability to oxygen and water.

Discussion and conclusion

The influence of water on oxidation reactions has been the subject of much study [15] that has led to revealing conclusions. The larger the amount of water food contains, the higher is reactant mobility and hence the rate of the reactions involved. As can be seen from Table II, the mean a_w values for all the samples studied were above 0.65 units after 5 days of storage; the increase in a_w was even more marked after the tenth day, with values of about 0.730. Because the presence of water at low a_w levels can delay oxidation owing to hydrogen bonding between water and hydroperoxides, oxidation should not occur to an appreciable extent at a_w values close to 0.50. The combined effect of accelerating and delaying factors therefore results in minimal or even decreased oxidation over the a_w range 0.400–0.500; above this upper limit, oxidation increases and eventually decreases again at $a_w > 0.850$, which can be ascribed to the presence of metal traces that form unreactive insoluble hydroxides. The inclusion of antioxidants in this type of product somehow hinders oxidation provided proper storage conditions are maintained; however, the additives have been reported to lose some efficiency in the presence of protein [16]. The labels for the feeds studied gave mean protein contents of about 31% in all cases.

Consequently, avoiding hydration of the product is one mandatory caution in order to avoid its typical oxidative rancidification.

pH values scarcely changed during storage; they ranged from 5.17 to 5.29 for all the samples throughout the period studied, with a standard deviation of 0.04 and a coefficient of variation of 0.85%.

Table III

Total count of mesophilic aerobic bacteria in pet feed during storage at 9 °C and 80% RH

Product	Sample No.	Storage time (days)				
		0	5	10	15	20
Ox croquettes	1-2	2.48	7.92	8.08	8.18	8.17
Chicken and rice croquettes	3-4	4.83	7.27	7.75	8.55	8.50
Growing formula croquettes	5-6	4.47	7.47	8.20	8.65	8.46
Chicken and cereal croquettes	7-8	4.40	>7.11(4)	8.32	8.46	8.76
Meat croquettes	9-10	4.69	7.04	7.67	8.16	7.49
Ox, veal and vegetable croquettes	11-12	4.60	7.62	8.61	8.50	8.42
Meat and fish croquettes	13-14	3.81	8.06	8.38	8.87	8.74
Trout, salmon and tuna croquettes	15-16	4.79	7.59	8.01	9.20	8.92
Tuna croquettes	17-18	3.90	7.51	7.85	8.02	8.15
Rabbit, liver and carrot croquettes	19-20	4.12	7.65	8.77	8.30	8.76

Table IV

Total count of fungi in pet feed during storage at 9 °C and 80 % RH

Product	Sample No.	Storage time (days)				
		0	5	10	15	20
Ox croquettes	1-2	2.09	4.79	7.43	8.39	9.59
Chicken and rice croquettes	3-4	3.75	5.00	8.52	9.19	8.37
Growing formula croquettes	5-6	3.58	5.10	7.67	8.71	9.62
Chicken and cereal croquettes	7-8	4.13	5.49	7.87	8.68	9.66
Meat croquettes	9-10	4.08	5.11	7.49	8.72	9.41
Ox, veal and vegetable croquettes	11-12	3.89	4.27	8.02	8.77	8.87
Meat and fish croquettes	13-14	4.76	5.02	8.96	8.07	8.93
Trout, salmon and tuna croquettes	15-16	4.13	4.76	9.56	9.16	8.84
Tuna croquettes	17-18	3.18	5.28	7.67	8.50	9.67
Rabbit, liver and carrot croquettes	19-20	4.16	5.36	8.13	8.55	8.67

Table V

Total count of yeasts in pet feed during storage at 9 °C and 80% RH

Product	Sample No.	Storage time (days)				
		0	5	10	15	20
Ox croquettes	1-2	2.71	5.68	5.79	7.63	8.81
Chicken and rice croquettes	3-4	2.56	5.08	6.05	8.35	8.48
Growing formula croquettes	5-6	3.59	6.20	6.87	7.71	8.62
Chicken and cereal croquettes	7-8	4.14	5.63	6.67	7.80	8.77
Meat croquettes	9-10	4.35	5.57	5.37	7.33	8.46
Ox, Veal and vegetable croquettes	11-12	2.69	5.35	6.53	7.61	8.42
Meat and fish croquettes	13-14	3.30	5.04	6.36	7.99	8.83
Trout, salmon and tuna croquettes	15-16	3.18	5.84	6.84	7.88	8.14
Tuna croquettes	17-18	3.18	6.18	6.17	8.36	9.30
Rabbit, liver and carrot croquettes	19-20	3.35	5.53	5.64	8.48	9.24

Regarding microbiological parameters (mesophilic aerobic bacteria, fungi and yeasts), Tables III-V show the counts obtained during the storage period. The initial counts for the three microorganism groups studied did not exceed 4.8 log CFU/g; the largest values corresponded to mesophilic aerobic bacteria. After 5 days of storage, the counts for the bacteria ranged from 7.04 to 8.06 log CFU/g (Table III), whereas those for fungi and yeasts were 4.27-5.49 (Table IV) and 5.04-6.20 log CFU/g (Table V), respectively.

As a_w increased from 0.750 to near 0.800 at 20 days (Table I), the counts of mesophilic aerobic bacteria and fungi changed similarly, as can be seen in Fig. 1, which shows the evolution of the means for the different counts during the storage period. The total fungal count decreased at 20 days. Yeasts counts departed somewhat from the previous two. Thus, while their number was similar to those of fungi from the beginning to the fifth day, their increase was much more gradual, with mean counts of 6.25 and 7.90 log CFU/g at 10 and 15 days, respectively. However, the trend was inverted at 20 days, where the counts of mesophilic aerobic bacteria were smaller (8.43 log CFU/g *versus* 9.26 log CFU/g for fungi and 8.70 log CFU/g for yeasts).

The water activity of foods is known to significantly influence the proliferation and metabolic activity of microorganisms including their toxin production and survival capacity [17-19]. Most food-related microorganisms can grow better at fairly high a_w values and only a few require a low water activity to develop. As a rule, fungi are more tolerant to a_w decreases than are yeasts, which in turn are more tolerant than bacteria.

Pet feed is stable against microbial growth on account of its low water activity ($a_w < 0.60$). Exposure of an open package to a high RH during storage gives rise to rehydration and increased a_w values up to 0.80 or even higher. This facilitates the development of yeasts (some of the genus *Saccharomyces*) and of fungi of the genera *Eurotium*, *Monascus*, *Aspergillus*, *Chrysosporium*, *Wallemia* and *Penicillium* [20].

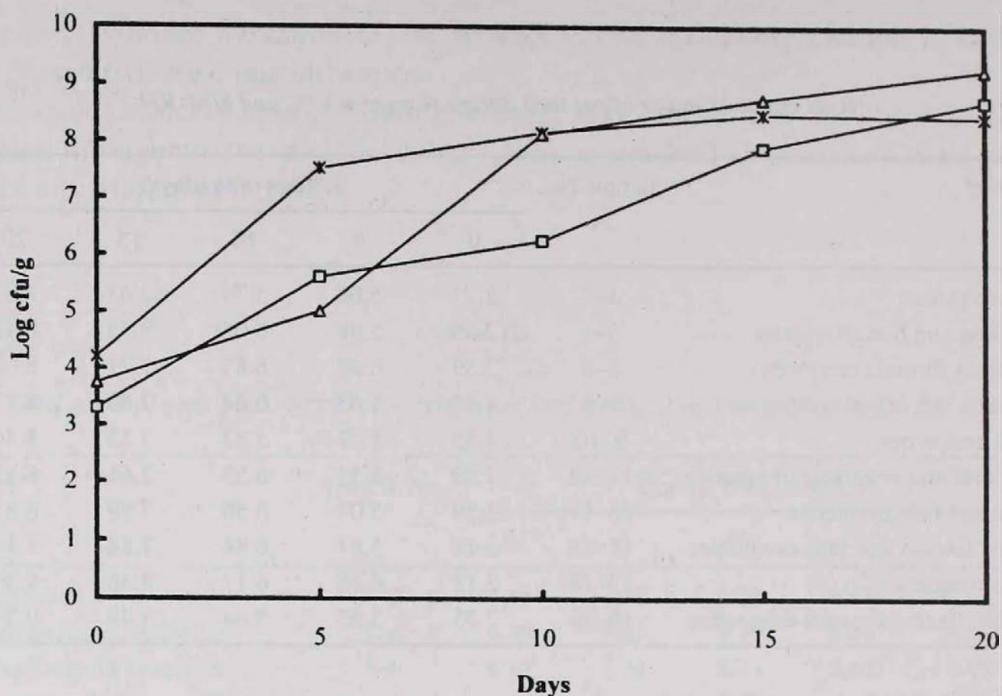


Fig. 1. Changes in the different counts (mean values) during the storage period

* Total viable count, Δ fungi, □ yeast

Some authors [17, 20, 21] have noted a risk of mycotoxins (basically penicillic acid, aflatoxin and ochratoxin A) being produced at low a_w values. In fact, mycotoxins can be formed in the typical a_w range for IMFs; however, precise determination of the minimum a_w values required for the production of the mycotoxins known to develop in pet feed would be required. A given mycotoxin is frequently produced by various species and genera (e.g. *Aspergillus* and *Penicillium*) with potentially different a_w requirements. Usually, toxigenic fungi grow at lower a_w values than those required for their toxins to be produced.

Careful moisture control is critical to ensure preservation of the quality of desiccated foods during storage. Quality losses here can result from browning basically of the non-enzymatic type, oxidation, nutrient losses and the course of microbial alteration. The Code of Federal regulations of the US Food and Drug Administration [22] prescribes that those foods where a_w must be controlled in order to prevent microbial growth should be processed in order to ensure maintenance of an adequate moisture level. One of the ways of ensuring that a_w cannot reach unsafe levels is by use of physical or other types of barriers intended to protect the food against excessive moisture.

Based on the results of this study, the stability of pet feed during its shelf life relies on storage under conditions where rehydration and the consequent increase in a_w and in the proliferation and development of detrimental germs – particularly fungi – are avoided.

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IN VITRO ANTIMICROBIAL PROPERTIES OF AZIDOTHYIMIDINE (AZT)*

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In addition to the activity against a number of retroviruses, azidothymidine (AZT) has antibacterial activity against many bacteria. The effect of AZT on 224 bacterial species, including 25 strains of *Salmonella* spp. isolated from HIV-positive patients, was tested. AZT had no activity against all the strains of tested Gram-positive bacteria and *Pseudomonas* species (MIC > 128 µg/ml), whereas a different activity against *Enterobacteriaceae* (MIC range, 128 to 0.06 µg/ml) was found. In particular 76% of *Salmonella* spp. isolated from HIV-positive patients showed MICs > 1 µg/ml, whereas similar MICs value were found in 50% of the *Salmonella* strains isolated from HIV-negative subjects. In addition, strains of *Salmonella* isolated from stools were more resistant to AZT when compared to strains isolated from blood even if this difference was not statistically significant. No correlation was found between length of therapy and *Salmonella* resistance to AZT in HIV-positive patients and a low incidence of *Salmonella* relapses in subjects treated with AZT was observed. The possibility that AZT may have an ancillary benefit in controlling some bacterial infections in AIDS patients is discussed.

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Azidothymidine (AZT) is a synthetic analogue of thymidine in which the 3'-hydroxyl group of thymidine has been replaced with a 3'-azido(-N₃) group. AZT is phosphorylated in cells to 5'-triphosphate by cellular enzymes. AZT triphosphate competes with thymidine triphosphate for reverse transcriptase of retrovirus in the incorporation into viral DNA. Once incorporated, AZT triphosphate acts as chain terminator, blocking DNA synthesis by susceptible human and animal retroviruses [1].

In addition to their antiviral activity, AZT possess bactericidal activity also against Gram-negative bacteria of the family of *Enterobacteriaceae*. This activity is exerted against organisms rich in thymidine kinase (TK), whereas bacteria lacking TK, such as *Pseudomonas aeruginosa* and *Mycobacterium avium*, are resistant to AZT [2]. However other factors, such as cell-wall permeability of the microorganism may affect the antibacterial activity of AZT.

The purpose of the present study was: i) To gain information on the in vitro antibacterial activity of AZT against bacteria isolated from clinical source. ii) To evaluate the in vitro activity of AZT against strains of *Salmonella* spp. isolated from HIV-positive or AIDS patients. iii) To evaluate the impact of AZT therapy in HIV-positive subjects on susceptibility of bacteria to AZT.

Materials and methods

AZT. Stock solutions of AZT (SIGMA Chemical Co., Italy) were prepared in sterile distilled water at concentration of 1280 µg/ml and sterilized by filtration. Solutions were freshly prepared for each day of the experiment.

Organism and culture condition. A total of 224 bacterial strains were tested. They were: *E. coli* (62), *Providencia* spp. (3), *Citrobacter* spp. (2), *Serratia marcescens* (3), *Morganella morganii* (2), *Proteus mirabilis* (4), *Enterobacter aerogenes* (1), *Enterobacter cloacae* (3), *Klebsiella pneumoniae* (9), *Klebsiella oxytoca* (1), *Edwardsiella* spp. (1), *Shigella sonnei* (1), *Shigella flexneri* (1), *Yersinia enterocolitica* (7), *Staphylococcus aureus* (36), coagulase negative staphylococci (7), *Enterococcus* spp. (11), *Streptococcus pyogenes* (2), *Streptococcus agalactiae* (5), *Pseudomonas aeruginosa* (9), *Burkholderia cepacia* (5), *Pseudomonas putida* (1), *Vibrio cholerae* (3), *Vibrio alginolyticus* (1), *Aeromonas hydrophila* (1), *Rhodococcus equi* (3), *Bacillus cereus* (1). In addition, 39 strains of *Salmonella* spp., 25 of which isolated from HIV-positive patients were also tested. All organisms were stored in skim milk at -70 °C and were subcultured twice on blood agar plates before susceptibility testing was done.

Determination of MICs and MBCs. MICs and MBCs were determined by a broth dilution technique according to that reported [3]. Serial twofold dilutions of AZT were performed in Mueller-Hinton broth (MHB) from 128 µg/ml to 0.06 µg/ml. Overnight growth of each strain was diluted in the MHB at a turbidity equal to a McFarland standard of 1.0 and then added to an equal volume (1 ml) of serial dilutions of AZT. A tube of MHB without AZT serves as a growth control. These mixtures were incubated statically at 37 °C for 24 h.

MICs values were recorded as the lowest AZT concentration at which no visible growth was seen. MBC determinations were performed by subculturing 0.01 ml samples from broths showing no visible growth and incubating them for 18-24 h at 37 °C. The MBC was defined as the lowest compound concentration at which >99.9% of the original bacteria were killed.

Statistics. Statistical analysis was carried out with the chi-square test with Yates' correction.

Results

We have examined a total of 224 bacterial strains isolated from clinical sources. The results suggest differences in sensitivity to antibacterial effect of AZT between Gram-positive and Gram-negative bacteria. In fact, AZT demonstrated poor activity against all Gram-positive bacteria tested ($\text{MIC} > 128 \mu\text{g/ml}$). As showed in Table I, among Gram-negative bacteria, AZT was not active against all strains of *Pseudomonas aeruginosa*, *Burkholderia cepacia* and *Pseudomonas putida* ($\text{MIC} > 128 \mu\text{g/ml}$). Two strains of *Vibrio cholerae* O1 (one of which isolated in Bari in the 1994) have a $\text{MIC} > 128 \mu\text{g/ml}$, whereas a strain of *V. cholerae* NAG and a strain of *Vibrio alginolyticus* showed MICs of 32 $\mu\text{g/ml}$ and 0.25 $\mu\text{g/ml}$, respectively. Eleven of 62 strains of *Escherichia coli* have a $\text{MIC} > 128 \mu\text{g/ml}$, 31 (50%) have a MIC value ranging from 64 $\mu\text{g/ml}$ to 2 $\mu\text{g/ml}$ and 20 (32.25%) have a $\text{MIC} \leq 1 \mu\text{g/ml}$.

Of the 39 strains of *Salmonella* tested, 5 (12.82%) showed $\text{MICs} > 128 \mu\text{g/ml}$, 20 (51.28%) showed MICs ranging from 64 $\mu\text{g/ml}$ to 2 $\mu\text{g/ml}$ and 14 (35.89%) showed $\text{MICs} \leq 1 \mu\text{g/ml}$. *Enterobacter cloacae* exhibited MICs in the range 32–1 $\mu\text{g/ml}$. The MICs of AZT of *Proteus mirabilis* was $\geq 128 \mu\text{g/ml}$ and 64 $\mu\text{g/ml}$ for two strains and 8 $\mu\text{g/ml}$ for the remaining two. AZT demonstrated poor activity also against strains of *Providencia*, *Serratia*, *Morganella*, *Citrobacter*, *Enterobacter* and *Shigella* tested. The seven strains of *Yersinia enterocolitica* showed MICs in the range from 128 $\mu\text{g/ml}$ to 16 $\mu\text{g/ml}$. In all cases MBCs were generally within twofold dilutions of the MICs (data not shown). Figure 1 gives information about the MICs value and the *Salmonella* spp. tested. In particular the 76.92% (10/13) of *S. enteritidis* tested, showed MICs value $> 1 \mu\text{g/ml}$. The 60% (3/5) of *S. typhi* showed $\text{MICs} > 1 \mu\text{g/ml}$, the 54.54% (6/11) of *S. typhimurium* showed $\text{MICs} > 1 \mu\text{g/ml}$. AZT was most active ($\text{MIC} = 0.125 \mu\text{g/ml}$) against two strains of the rough mutants *S. minnesota* Rhn 202 (Rc) and R595 (Re) of our collection (data not shown). This fact may support the concept that other factors, such as cell wall permeability of the organism, may also affect the antibacterial activity of AZT.

The effect of AZT on *Salmonella* strains isolated from HIV-positive and HIV-negative subjects is also showed in Fig. 1. As can be seen, the 76% (19/25) of *Salmonella* spp. isolated from HIV-positive patients showed $\text{MICs} > 1 \mu\text{g/ml}$ whereas similar MICs value were found in the 50% (6/12) of the *Salmonella* strains isolated from HIV-negative subjects ($p > 0.05$). $\text{MICs} \leq 1 \mu\text{g/ml}$ were found for the 24% (6/25) of *Salmonella* spp. isolated from HIV-positive subjects and for 50% (6/12) of *Salmonella* spp. isolated from HIV-negative subjects.

As can be seen in Fig. 2 we have found $\text{MICs} > 1 \mu\text{g/ml}$ for the 77.8% (14/18) of *Salmonella* spp. isolated from stools. The 60% of *Salmonella* strains isolated from blood showed similar MICs values ($p > 0.05$).

Table I

MICs ($\mu\text{g/ml}$) of AZT against Gram-negative clinical isolates

Gram-negative	*	>128	128	64	32	16	8	4	2	1	0.5	0.25	0.125	0.06
<i>A. hydrophila</i>	1	1												
<i>Citrobacter</i> spp.	2	1			1									
<i>E. coli</i>	62	11		1	1	6	5	13	5	12	6	1		1
<i>E. aerogenes</i>	1	1												
<i>E. cloacae</i>	3				1	1				1				
<i>Edwardsiella</i> spp.	1					1								
<i>K. pneumoniae</i>	9					2	1		3	2		1		
<i>K. oxytoca</i>	1	1												
<i>M. morgani</i>	2	2												
<i>P. mirabilis</i>	4	1	1	1			1							
<i>P. alcalifaciens</i>	1	1												
<i>P. rettgeri</i>	1	1												
<i>P. stuartii</i>	1	1												
<i>P. aeruginosa</i>	9	9												
<i>B. cepacia</i>	5	5												
<i>P. putida</i>	1	1												
<i>Salmonella</i> spp.	34	5		1	1		2	4	9	4	5	1	2	
<i>S. typhi</i>	5						1	1	1		2			
<i>S. marcescens</i>	3	3												
<i>S. flexneri</i>	1							1						
<i>S. sonnei</i>	1	1												
<i>V. alginolyticus</i>	1											1		
<i>V. cholerae</i> O1	2	2												
<i>V. cholerae</i> NAG	1				1									
<i>Y. enterocolitica</i>	7		1		1	5								

*Number of strains examined

Discussion

AZT is widely used for the treatment of HIV infection. AZT therapy appears to partially restore immune function in some patients and reduce both the frequency and severity of opportunistic infections that are responsible for the mortality of AIDS patients [4]. Several studies have dealt with the antibacterial effect of AZT. Elwell et al. [2] were the first to report a potent bactericidal activity of AZT against *E. coli*, *S. typhimurium*, *K. pneumoniae*, *S. flexneri* and *E. aerogenes*. No activity was found against *P. aeruginosa*, Gram-positive bacteria, *M. tuberculosis*, anaerobic bacteria and most fungal pathogens.

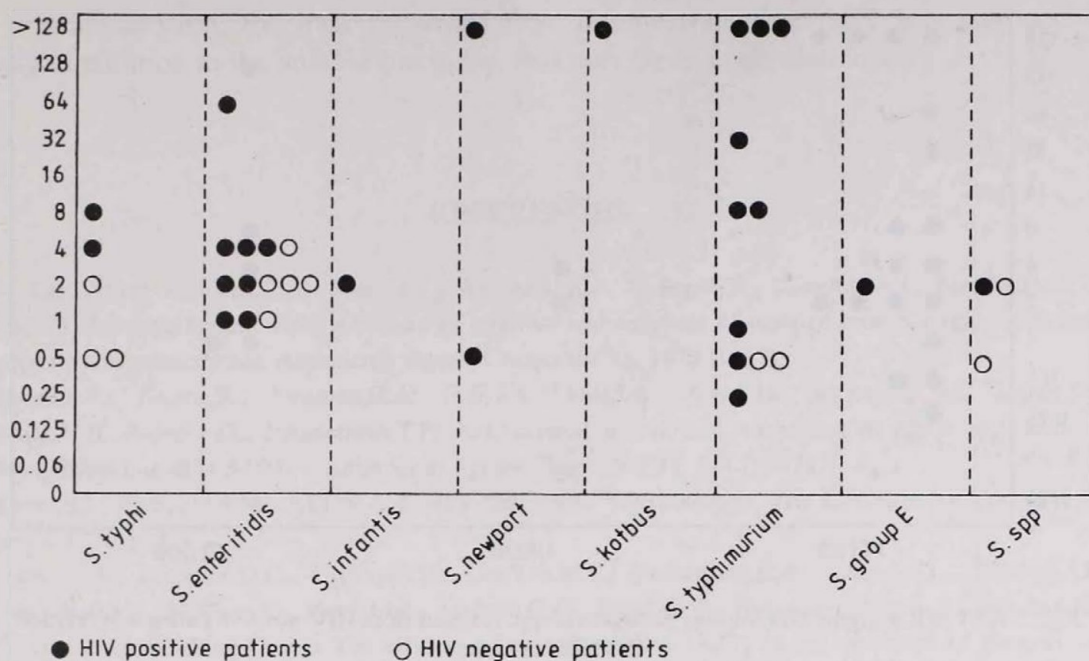


Fig. 1. AZT MICs ($\mu\text{g/ml}$) for various *Salmonella* species isolated from HIV-positive and HIV-negative patients

Our data are in accordance with what reported by these authors for Gram-positive bacteria and for *P. aeruginosa*. Saito and Tomioka [5] have reported that these organisms lack TK activity which may explain their resistance to AZT. Another hypothesis is that the different chemical composition of the cell-wall of Gram-positive bacteria may affect the permeability to AZT. In addition, MICs found by other authors for *Enterobacteriaceae* are lower than MIC values found in our study [2, 9, 10]. If this represents a result of a different distribution of serotypes of these bacteria in our country or an increase of resistance to AZT as result of widely use of this drug, remains to be established. Another intriguing finding is that AZT may act synergically with trimethoprim in vitro against strains of Gram-negative bacteria including *Salmonella*, *Shigella*, *Klebsiella* and *Proteus* [2]. However, in vivo studies have showed that AZT is effective in the treatment of experimental infections by *E. coli* and *S. dublin* in mice and calves, respectively [6]. Bacterial protein and RNA synthesis, as well as bacterial cell division, seem to be required for the bactericidal activity of AZT against *E. coli* and *S. typhimurium* that were susceptible at a concentration of AZT of 1 $\mu\text{g/ml}$ [7]. This is the clinically achievable plasma concentration of AZT after therapeutic doses of 1,200 mg/day [8]. All these studies have supported the possibility that AZT might also control bacterial infections in HIV-positive patients treated with this drug. In this respect, Salmon et al. [9] reported that AZT treatment alone is efficient in preventing relapses of *Salmonella* infections and suggested that antimicrobial maintenance therapy might not be necessary when patients are treated with >400 mg/day of AZT.

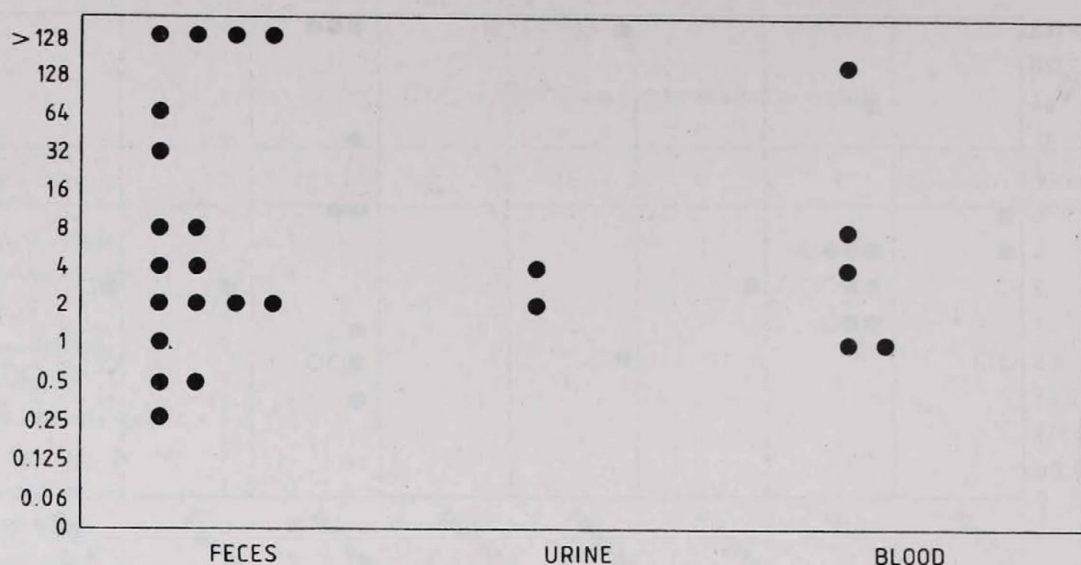


Fig. 2. AZT MICs ($\mu\text{g/ml}$) for various *Salmonella* spp. isolated from HIV-positive patients in relation to source of isolate

The recent decrease of *Salmonella* infections observed, also in our experience, in HIV-positive patients may be attributed, in addition to the *Pneumocystis carinii* prophylaxis with trimethoprim, also to the widespread use of AZT. In this respect, Sperber et al. [10] have demonstrated that at concentration achieved during the treatment of HIV-infection, AZT has activity against *Salmonella* spp., whereas didanosine and zalcitabine at currently used dosages, seem to have no clinical usefulness in the treatment of *Salmonella* infections.

However, the emergence of AZT resistance has been demonstrated in vitro for *E. coli* and *S. typhimurium* [2] due to a lack of significant levels of TK. The emergence of resistance has also been demonstrated in vivo in patients studied by Salmon et al. [9].

The possibility that antiviral therapy with AZT could be effective in the prevention or treatment of *Salmonella* infections is also supported by the evidence that AZT is also effective against *Salmonella* within the macrophage cell line J 774-2 [11].

Recently Tocalli et al. [12] have reported that strains of *Salmonella* isolated from 1987 to 1991 both from HIV-positive and HIV-negative subjects showed MICs to AZT ≤ 1 $\mu\text{g/ml}$ suggesting that the widespread use of AZT did not lead to a more frequent occurrence of AZT-resistant *Salmonella* strains in HIV-positive subjects. Our data show that 76% of *Salmonella* isolated from HIV-positive patients have a MIC to AZT > 1 $\mu\text{g/ml}$, whereas 50% of *Salmonella* isolated from HIV-negative patients have equal MIC values. In our experience no correlation was found between length of therapy and *Salmonella* resistance to AZT in HIV-positive patients (data not shown). However, MICs > 1 $\mu\text{g/ml}$ were also found in the majority of HIV-positive patients that have not received AZT. We have observed a low incidence of *Salmonella* relapses in subjects treated with AZT, even if in some patients a concomitant antibacterial therapy, as trimethoprim, and/or the immunologic effects of AZT may account for this observation.

In conclusion, the data presented here demonstrate that AZT has antibacterial activity in addition to the anti HIV activity, that may have a role also in vivo.

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INHIBITORY EFFECT OF PENTOXIFYLLINE ON HLA-DR EXPRESSION AND GLYCOSAMINOGLYCAN SYNTHESIS OF RETROBULBAR FIBROBLASTS INDUCED BY INTERFERON GAMMA

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Glycosaminoglycan (GAG) accumulation produced by retroocular fibroblasts (REF) has been observed in patients with thyroid-associated ophthalmopathy (TAO). Various cytokines are able to express HLA-DR molecules and stimulate the REF to proliferate GAG and free oxygen radicals. Pentoxifylline (Ptx) is known to have complex immunomodulatory effects on production of cytokines including interferon gamma (IFN-gamma). Ptx has been assumed to inhibit the cytokine-induced production of GAG and HLA-DR expression. We wished to determine whether Ptx has an effect on the IFN-gamma induced HLA-DR expression and influences the spontaneous and cytokine-induced GAG synthesis of REF. REF derived from extraocular muscles of healthy subjects were cultured without and with IFN-gamma. The effect of Ptx on expression of HLA-DR molecules and the production of GAG by REF was determined.

Glycosaminoglycan was measured by incorporation of (³H)glycosamine into GAG. HLA-DR expression was analysed by fluorescence activated cell sorter. IFN-gamma (50, 100 and 500 U/ml) induced an increase in expression of HLA-DR molecules of REF. Ptx was proved not to be toxic for cultured cells. This drug was able to dose-dependently inhibit HLA-DR expression of REF. Both spontaneous and IFN-gamma-induced GAG synthesis of REF was inhibited by Ptx (100, 500 and 1000 mg/l, respectively). Due to in vitro inhibitory effects, Ptx is potentially able to modify the antigen presentation and the GAG synthesis by REF and it might be a useful therapeutical drug in the treatment of TAO.

Thyroid associated ophthalmopathy (TAO) is considered to be a genetically determined autoimmune disorder characterized by infiltration of lymphocytes and enlargement of extraocular muscles, accumulation of glycosaminoglycan (GAG) resulting in clinical manifestation of edema, proptosis, diplopia and optical nerve compression [1–

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5]. The activated lymphocytes have been shown to secrete several cytokines including tumor necrosis factor alpha (TNF-alpha), interleukin-1 (IL-1) and interferon gamma (IFN-gamma) which are able to express HLA-DR antigens and stimulate fibroblasts to proliferate, produce GAG and free oxygen radicals [6-9]. Anti-cytokine antibodies, IL-1 receptor antagonist were able to inhibit the GAG synthesis of retroorbital fibroblasts (REF) [15]. Furthermore, recently it was observed that superoxide radicals generated in culture media were able to stimulate retroocular fibroblasts. Methimazole as a free radical scavenger drug inhibited superoxide-induced proliferation in a dose-dependent manner [10].

Pentoxifylline (Ptx) is known to have a complex immunomodulatory effect mediated by regulation of cytokine production [11-13]. Therefore, it is assumed that Ptx might have influence on cytokine mediated GAG production. We addressed two questions. First, what is the relative potency of Ptx on IFN-gamma induced HLA-DR expression on REF? Second, can Ptx suppress the spontaneous and IFN-gamma-induced GAG production of REF?

Materials and methods

Human fibroblast culture. REF were obtained from patients undergoing strabismus correction. Tissue specimens were minced and placed in Petri dishes and cultured in tissue culture medium in Dulbecco's minimal essential medium (DMEM) with penicillin 100 U/ml, gentamycin (20 µg/ml), amphotericin (1.25 µg/ml) supplemented with heat inactivated FBS (20% vol/vol). The cultures were maintained at 37 °C in a humidified incubator (95% air, 5% CO₂). After 5-6 days fibroblasts grew from the explants and when a near-confluent monolayer had developed the cells were passed using trypsin-EDTA. Fibroblasts were transferred into triplicate 24-well plates at a density of 1×10^5 cells/well. Only cells derived from passage 4-6 were used. Cultures were incubated for three, five and ten days with IFN-gamma (gift of Hoffmann La Roche produced by Genentech, San Francisco, USA) 50, 100 and 500 U/ml, respectively. Cell viability of cultures of three, five and ten days was determined by trypan blue exclusion test and found to be 88, 76 and 70%, respectively.

Investigation of HLA-DR expression of REF. HLA-DR expression was investigated as published elsewhere [14]. Briefly, DR expression was tested initially (day 0) and days 3, 5 and 10 of cultures in the presence and absence of IFN gamma and Ptx (Biogal, Debrecen, Hungary) of various concentrations. For indirect immunofluorescence, cells were resuspended in 200 µl RPMI containing 10% FCS 10 mM HEPES and were incubated for 30 min at 4 °C with 5 µl monoclonal anti-DR framework antibody (DAKO). The cells were washed twice and the pellet incubated for 30 min at 4 °C with 1:100 dilution of FITC conjugated rabbit anti-mouse immunoglobulin (Cooper Biomedical Inc., Penn). After two washes the cells were analysed by fluorescence activated cell sorter (FACS II Beckton-Dickinson Co., Sunnyvale, Calif.). At least 10^5 cells sample were sorted.

Assay of GAG synthesis. Determination of GAG synthesis was measured by a previously described technique [15]. The GAG production by the confluent fibroblasts was determined by incorporation of (³H)glycosamine into GAG. The cultures were labelled with 1 µCi of (³H)glycosamine (26 Ci/mmol). At the end of the incubation 0.1 ml of DMEM containing 5.0 g/l pronase (Sigma) was added to each well. After the cell detached 0.4 ml of a suspension containing both cells and medium was placed in 2.0 ml Eppendorf tubes and incubated at 55 °C for 4 h. After incubation the suspension was centrifuged and the supernatant was placed into tubes. A GAG carrier consisting of 0.1 ml of a solution of hyaluronic acid (1.0 g/l) and chondroitin sulphate (1.0 g/l) (Sigma) and 1.0 ml of 1%

cetylpyridinium chloride (CPC) in 0.002M Na₂SO₄ was added to precipitate GAG. After 20 min at room temperature the solution was filtered through a Whatman GF/F glass fibre filter. The filters were washed twice with 1.0 ml of 0.1% CPC in 0.5 M NaCl and four times in distilled water, then placed in scintillation vials and dried in an oven at 40 °C. Radioactivity was measured by a liquid scintillation counter (Nuclear Chicago Isocap 300). Each assay was performed in triplicate and the mean results were expressed as cpm of (³H)GAG per 10³ cells.

Measurement of luminol-dependent chemiluminescence. Horseradish peroxidase activity was measured by our previously published method [16]. Briefly, the effect of Ptx on horseradish peroxidase (Sigma) was tested by adding 2×10^{-5} M luminol, variable concentrations of Ptx (10, 100, 500 and 1000 mg/l, respectively), and 100 μ mol of hydrogen peroxide. The reaction was started by addition of 1.0 μ g of horseradish peroxidase in 2.0 ml to the mixture at room temperature and the light emission was integrated for 15 s by a LKB luminometer (LKB2210). The peroxidase-independent chemiluminescence reaction was tested as above except for the absence of horseradish peroxidase. To a 2.0 ml vial 2×10^{-4} M luminol, 10 mmol of hydrogen peroxide were added. The effect of various concentrations (from 10 to 1000 mg/l) of Ptx was tested and contrasted to results obtained in absence of Ptx.

Statistics. A multiple comparison method (*t*-test) was used for statistical analysis using the ANOVA program (Sigma Star statistical software).

Results

Effect of Ptx on HLA-DR expression. We found a dose-dependent effect of IFN-gamma on HLA-DR expression. 50 U/ml, 100 U/ml and 500 U/ml of IFN-gamma induced a $25 \pm 6\%$, $45 \pm 12\%$ and $62 \pm 17\%$ increase, respectively, in the number of DR positive cells. To test the possible effect of Ptx on IFN gamma induced DR expression we used in each culture 100 U/ml of IFN gamma. We observed that Ptx alone (10, 50, 100, 500, 1000 mg/l) had no effect on spontaneous expression of DR molecules in culture of REF (data not shown). Surprisingly, Ptx dose-dependently inhibited the IFN-gamma induced HLA-DR expression on REF (Fig. 1). 100, 500 and 1000 mg/l of Ptx resulted in a significant reduction DR expression. This inhibitory effect cannot be attributed to the possible cytotoxicity of the drug since the viability of cells was not changed remarkably on cultures at day 3 (from 88 ± 8 to 81 ± 6.5), day 5 (from 76 ± 6.5 to 72 ± 6.2) and day 10 (from 70 ± 11 to 64 ± 8.9) in the presence of 1000 mg/l of Ptx. To test the possible free radical scavenging effect of Ptx we measured in the peroxidase-dependent and independent chemiluminescence in cell free conditions. Ptx had no effect on either horseradish peroxidase-dependent and peroxidase-independent chemiluminescence (data not shown).

Effect of Ptx on GAG synthesis. We observed that relatively high doses of Ptx (500 and 1000 mg/l) significantly decreased the spontaneous GAG synthesis of fibroblasts (Table I). IFN-gamma (100 U/ml) induced a remarkable increase in GAG production of REF cultures (Fig. 2). Both spontaneous and IFN-gamma-induced GAG synthesis was significantly inhibited by 100-1000 mg/l of Ptx. At the highest concentration (1000 mg/l) of Ptx the enhancing effect of IFN-gamma on GAG synthesis was virtually abolished.

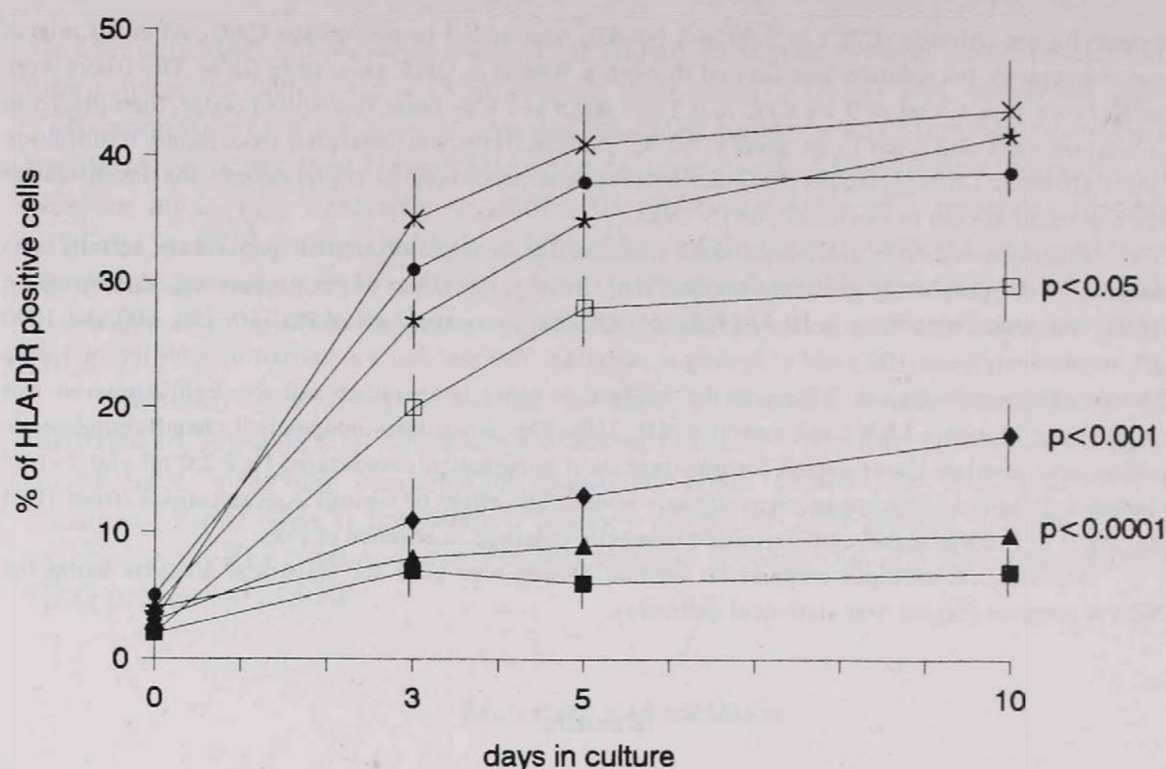


Fig. 1. Effect of Pentoxifylline on the HLA-DR expression of retroorbital fibroblasts induced by interferon gamma (100 U/ml).

Symbols and bars indicate the mean and standard deviation of triplicate cultures. Crosses: fibroblasts with IFN-gamma of 100 U/ml without Ptx. Dots: fibroblasts with IFN-gamma of 100 U/ml with Ptx of 10 µg/l.

Asterisks: fibroblasts with IFN-gamma of 100 U/ml and Ptx of 100 µg/l. Squares: fibroblasts with IFN-gamma 100 U/ml and Ptx of 500 µg/l. Diamonds: fibroblasts with IFN-gamma of 100 U/ml and Ptx of 1000 µg/l. Triangles: fibroblasts without IFN-gamma and Ptx.

Filled squares: fibroblasts without IFN-gamma and with Ptx of 1000 µg/l

Discussion

Predominance of Th1 profile of cytokine production was found in retroorbital tissue of Graves' patients [17]. Cytokines – including IFN-gamma – exert several effects that may be important in the induction and perpetuation of autoimmune processes. Although these immunological investigations have improved our knowledge on the pathomechanism of TAO, we remained limited in our ability to treat this disease effectively. Various therapeutical interventions have known to modify the production of cytokines [18]. However, intravenous methylprednisolone and immunoglobulin therapies have potential serious side effects [18]. Therefore, it is necessary to find new cytokine antagonists interfering with cytokine synthesis, receptor binding or signal transduction.

Table I

Effect of Pentoxifylline (Ptx) on spontaneous ^3H -glycosamine incorporation into glycosaminoglycan produced by retroorbital fibroblast cultures

Ptx (mg/l)	Culture time (days)	
	3 (n=5) (c.p.m.)	5 (n=5) (c.p.m.)
0	485+62	506+51
10	470+74	562+68
100	365+68	488+47*
500	242+48*	280+65**
1000	154+42**	199+44**

* = $p < 0.05$

** = $p < 0.001$

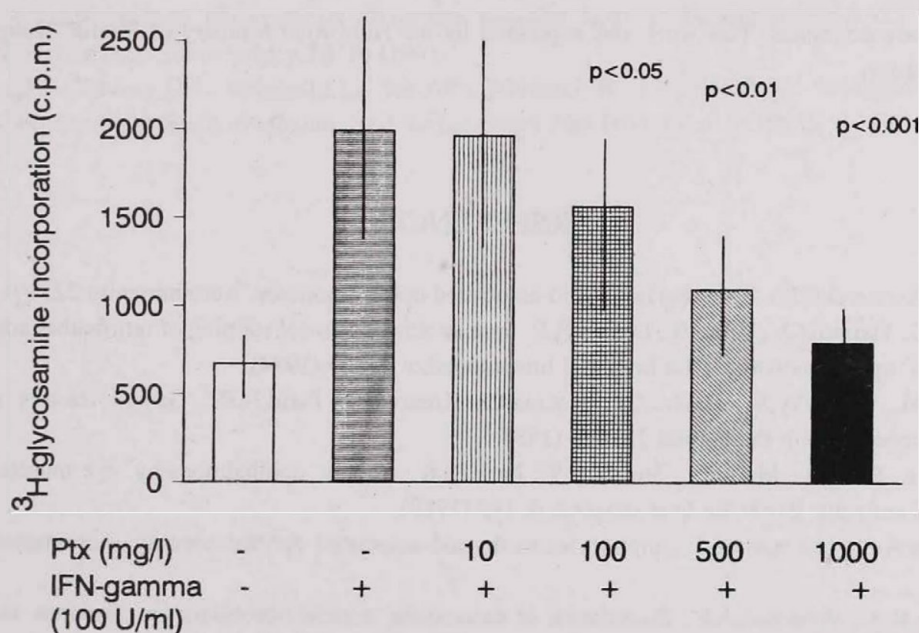


Fig. 2. Effect of Pentoxifylline on GAG synthesis of retroorbital fibroblasts induced by IFN-gamma(100 U/ml). The bars show the synthesis of fibroblasts on day 5 calculated from triplicate cultures of 5 experiments. The lines indicate the standard deviations.

Ptx, a methylxantine derivate and phosphodiesterase inhibitor is known to inhibit the production of TNF-alpha and IL-1 [19]. We found that Ptx had no cytotoxic effect on REF and it influenced the expression of HLA-DR molecules. Ptx was able to dose-dependently inhibit HLA-DR expression induced by IFN-gamma. The exact mechanism

of this phenomenon is not known. It was assumed that similarly to the inhibitory effect of Methimazole on HLA-DR expression, Ptx has a free radical scavenging potential. This suggestion could not be supported, because we could not detect remarkable free radical scavenging effect of high concentrations of Ptx even in high concentrations. Therefore, we hypothesized that the influence of Ptx on HLA-DR expression might be mediated by increasing of intracellular cAMP of REF and modification of signaling [20].

Our study demonstrated that in vitro exposure to Ptx by itself resulted in a dose-dependent inhibition of GAG synthesis at high concentration (1000 mg/l). This inhibitory effect proved to be more apparent in the cytokine induced GAG production. The inhibitory effect of Ptx might be mediated by different pathways. (i) Ptx is able to directly decrease the production of GAG by fibroblasts [19]. (ii) Cytokines' (TNF-alpha, IFN-gamma, IL-1) induced signaling is modified by increasing intracellular cAMP [20]. (iii) Ptx can modify the cytokine antagonist gene expression [21].

Our in vitro studies show that Ptx enables to decrease the accumulation of REF, it is impossible, however, to extrapolate this observation to conditions occurring in vivo in Graves' patients. Whatever mechanisms exist, the validity of this in vitro study can only be confirmed in ongoing prospective clinical studies.

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SHAKE CULTURE STUDIES FOR THE PRODUCTION OF AMYLASES BY *THERMOMYCES LANUGINOSUS*

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The paper describes shake flask culture optimization studies for the production of amylases by *Thermomyces lanuginosus*. The culture was found to produce maximally alpha-amylase and glucoamylase (18.4 & 11.2 units/ml), respectively when the medium contained rice flour (2% w/v) as carbon and corn steep liquor as nitrogen source with medium pH adjusted to 5.5 and incubated for 72 h under shaking conditions (150 rpm) at 50 °C.

Thermomyces lanuginosus a thermophilic fungus has been documented for amylolytic activity [1, 2] including glucoamylase [3, 4], alpha-amylase and alpha-glucosidase [5]. Although, the purification and characterization of alpha-amylase and glucoamylase has been described [4, 5], no report on the optimization of the enzyme production by *T. lanuginosus* is available. This study was carried out using a soil isolate of *T. lanuginosus* for optimizing the production of alpha-amylase and glucoamylase in shake flask culture.

Materials and methods

Microorganisms. The organism (*T. lanuginosus*) was isolated from soil sample [6] and maintained on potato dextrose agar medium slopes at room temperature and was subcultured fortnightly.

Medium for shake flask cultures. The production medium contained (g/l) soluble starch, 15.0; yeast extract, 4.0; $K_2HPO_4 \cdot 3H_2O$, 2.3; KH_2PO_4 , 2.0; $MgSO_4 \cdot 7H_2O$, 0.5; citric acid, 0.57; and 0.1 ml trace element solution [7]; the pH was adjusted to 5.0 using H_2SO_4 (0.1 N). In the experiments to study the effect of carbon sources, starch was replaced by the same amount of the following carbon sources: singhara flour (*Trapa* sp.), maize flour, wheat flour, wheat bran, rice flour and potato starch. For studying the effect of nitrogen sources yeast extract was replaced by NH_4NO_3 , $(NH_4)_2HPO_4$, Corn Steep Liquor (CSL), malt extract and urea in concentrations equivalent to the amount of nitrogen in

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yeast extract. All the experiments were carried out at flask level in triplicate and the mean standard error (M.S.E) at 5% level was calculated.

Inoculum. The conidial inoculum was obtained by scraping the aleurospores from the surface of agar slant culture (7-days-old culture) and suspending in 10 ml of Tween 80 (0.2%; v/v). Initial number of aleurospores was counted using a haemocytometer. Each shake flask culture was inoculated with 1 ml of this suspension containing 3×10^5 spores.

Culture conditions. Shake flask fermentations were performed in 250 ml Erlenmeyer flasks each containing 100 ml medium in an environmental shaker (New Brunswick Scientific model G-24 Edison, N.J., USA) at 50 °C and 150 rpm.

Determination of biomass. The culture was filtered through preweighed filter paper and was dried in an oven at 60 °C for 24 h or till a constant weight was achieved and again weighed.

Analytical procedures. Alpha-amylase (dextrinizing) activity was measured by the iodine method. The reaction mixture containing 0.5 ml each of suitably diluted enzyme and soluble starch (1%, w/v) was incubated at 50 °C for 10 min and then the reaction was stopped immediately by adding 1 ml of 1 N HCl to the reaction mixture. After thoroughly mixing, 0.5 ml of I_2 solution (0.15% I_2 + 0.5% KI) was added to develop the colour and the absorbance was read at 550 nm using a DU-64 spectrophotometer. The residual starch was determined from the standard curve and the activity in the crude extract was estimated. The glucoamylase activity was analysed by measuring the glucose equivalents released from starch during the enzyme-substrate reaction using the DNS method [8]. The protease (acidic) activity was assayed by the method described by Sandhu et al. [9].

Enzyme units. The amount of enzyme required to hydrolyse one mg starch per minute at 50 °C has been defined as the alpha-amylase unit. The glucogenic activity is defined as the amount of enzyme required to release one micromole of glucose equivalent per minute under assay conditions as measured by DNS. The protease activity unit was expressed as the amount of enzyme required to release one micromole of tyrosine per minute under assay conditions.

Results

Effect of carbon source. The results in Table I show that wheat bran was able to support maximum growth of the organism (680 mg dry wt/flask). However, the maximum alpha-amylase (11.94 units/ml) and glucoamylase (3.42 units/ml) activity were attained in the medium containing rice flour. This was followed by singhara flour and wheat flour, respectively.

Effect of nitrogen sources. Of all the nitrogen sources tested in combination with starch as carbon source, corn steep liquor was found to be the most suitable source that resulted in very high yields of biomass (1565 mg dry wt/flask), alpha amylase (13.0 units) and glucoamylase (1.94 units/ml). The inorganic nitrogen sources $(NH_4)_2H_2PO_4$ and NH_4NO_3 were found to support growth and enzyme production almost equivalent to the yeast extract (Table II).

Effect of pH. The results in Table III show that an acidic pH (5.5) was suitable for the production of alpha-amylase (15.7 units/ml) and glucoamylase (10.7 units/ml). The lowering of the medium pH resulted in decreased amylase production.

Amylase production profile under optimized conditions. The amylase production was found to be maximum after 72 h of incubation where maximum alpha amylase (18.4 units/ml) and glucoamylase (11.8 units/ml) were expressed. However, this period was also

marked with the highest expression of protease activity (0.67 units/ml). There was a marked decrease in the protease activity thereafter and no protease activity was observed after 120 h (Table IV).

Table I

Effect of carbon sources on the production of amylases by T. lanuginosus

Carbon source	Biomass (dry wt, mg/flask)	Alpha-amylase (units/ml)	Glucoamylase (units/ml)
Singhara flour	445±22	10.4±0.21	3.0±0.13
Maize flour	355±34	5.7±0.32	1.7±0.09
Wheat flour	465±26	10.3±0.14	2.6±0.17
Wheat bran	680±12	2.5±0.22	1.8±0.16
Rice flour	500±34	11.9±0.11	3.4±0.31
Potato starch	270±14	0.9±0.12	1.7±0.07

Culture conditions: Working volume 100 ml; incubation time 5 days; temperature 50 °C; agitation rate 150 rpm; each carbon source added at 15g/l. S.E. at 5% level

Table II

Effect of nitrogen sources on the production of amylases by T. lanuginosus

Nitrogen sources	Biomass (dry wt, mg/flask)	Alpha-amylase (units/ml)	Glucoamylase (units/ml)
(NH ₄) ₂ SO ₄	260±22	1.0±0.09	0.1±0.001
NH ₄ NO ₃	360±56	4.1±0.16	0.1±0.003
(NH ₄)H ₂ PO ₄	265±34	3.8±0.23	0.2±0.003
Yeast extract	320±11	3.2±0.24	0.5±0.07
Corn steep liquor	1565±110	13.0±0.63	1.9±0.09
Malt extract	625±31	0.8±0.12	0.2±0.01
Urea	285±26	0.1±0.006	0.3±0.05

Culture conditions. Same as in Table I, except yeast extract has been replaced by the above-mentioned nitrogen sources and soluble starch was used as carbon source. S.E. at 5% level

Table III

Effect of pH on the amylases production by T. lanuginosus

pH	Biomass (dry wt, mg/flask)	Alpha-amylase (units/ml)	Glucoamylase (units/ml)
4.0	1035+89	4.2+0.31	0.4+0.17
4.5	1330+113	5.1+0.25	1.5+0.21
5.0	1595+145	10.2+0.67	6.1+0.14
5.5	1585+194	15.7+0.88	10.7+0.74
6.0	1495+85	13.4+0.54	10.3+0.36

Culture conditions. Same as in Table I, except rice flour, 2 % (w/v) as carbon source and corn steep liquor as nitrogen source. S.E at 5% level

Table IV

The amylase production profile of T. lanuginosus under optimized conditions

Incubation period	Biomass (dry wt, mg/flask)	Alpha-amylase (units/ml)	Glucoamylase (units/ml)	Protease (units/ml)
24	550+27	1.2+0.31	0.22+0.03	0.22+0.33
48	820+32	10.0+0.30	9.6+0.13	0.62+0.11
72	1580+52	18.4+0.62	11.8+0.26	0.67+0.07
96	1550+23	16.2+0.23	11.0+0.12	0.28+0.13
120	1320+54	15.7+0.13	10.7+0.18	—
144	940+43	8.9+0.33	5.9+0.78	—

Culture conditions. Working volume, 100 ml; rice flour, 2% (w/v); CSL as nitrogen source; temperature 50 °C; pH 5.5; agitation rate, 150 rpm. S.E at 5% level

Discussion

The pattern of amylase production observed in the present studies is similar to that in earlier reports [1, 5]. However, our studies have shown that using a spore suspension was effective for achieving high biomass in contrast to the findings of Hassum et al. [4] who suggested pregerminated conidial suspension for achieving high biomass. Starch may be easily replaced by rice flour as it was found to be suitable for enzyme expression, whereas, wheat bran supported maximum growth of the organism. The ability of a particular carbon source to induce high growth or enzyme production varies from source

to source [10]. Corn steep liquor was found to be the best nitrogen source for supporting high growth and maximum enzyme production. However, inorganic nitrogen sources $(\text{NH}_4)_2\text{HPO}_4$ was found to support glucoamylase expression as good as yeast extract as also observed by Hassum et al. [4], whereas for the expression of alpha amylase $(\text{NH}_4)\text{NO}_3$ was found to be suitable. The expression of enzymes when CSL was used as nitrogen source was almost four times higher than that achieved with yeast extract. The combination of rice flour and CSL was the best source for balanced enzyme expression. A slightly acidic pH (5.5) seems to support high amylase expression as also observed by Chen et al. [11], however, higher acidic pH affected the enzyme production appreciably. The production profile of amylases under optimized conditions clearly indicate that the biosynthesis increases up to 72 h and the decrease thereafter may be attributed to protease activity as observed in the present study. However no protease activity was observed after 96 h and the associated decrease in the activity may be ascribed to the cell lysis as observed earlier [4]. Further work on the localization of these enzymes during lag and exponential phase of growth and its regulation is currently under study.

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STUDIES ON CELLULASES OF *ASPERGILLUS* *NIDULANS* MUTANTS

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Mutants were characterized as to their morphology, auxotrophy and cellulase production. No apparent correlation was evident among these characters. The degree of repression or enhancement of the three components of the cellulase enzyme in the mutants varied considerably, indicating their separate genetic control. Considerable variations in the enzyme profiles of the mutants were observed as compared to the wild type. Enzyme production was also found to be related to development.

The bioconversion of biomass for the production of fuels and chemicals has recently become the focus of attention. Much attention is being paid to the enzymatic saccharification of cellulose, one of the most abundant natural source, as a potential source of energy. The most effective method for increasing the yield of cellulose enzymes has been the use of induced mutations followed by the selection of improved strains [1]. Though the yields of cellulases produced by *Aspergilli* are not comparable to some of the best-known cellulolytic fungi e.g. *Trichoderma* species [2, 3], *Aspergillus nidulans* which is known to be a good producer of β -glucosidase, gains importance by virtue of its being amenable to standard genetic analysis [4]. In the present study mutants of *A. nidulans* were studied to select for commercially useful hyperproducer strains as well as for future use in genetic crosses.

Material and methods

Strains. Wild type strain of *A. nidulans* (ATCC 62041) was isolated from soil. Some of the mutants used in the present study were obtained from the Microbial Type Culture Collection, Chandigarh. Some mutants were induced by UV. Conidia were suspended in sterile water (100/0.1 ml) and plated on Vogel's complete medium [5]. The plates were immediately treated with UV irradiations (Philips TUV, 30 watts) to obtain survival rate in the range of 8% to 20%. The survivors of UV

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treatment were transferred to MM plates and all those colonies which exhibited no growth on MM or evinced change in the colony colour were selected for further characterization. The selected mutants were characterized with respect to their cellulase production, auxotrophy and morphology. Mutants exhibiting less than 30% of the enzyme activity of the wild type were considered as hypo-producers for that enzyme and were designated as negative mutants.

Enzyme production. For estimating the cellulase activities of the mutants and wild type, they were grown in liquid broth. Fifty ml of Vogel's complete medium [5] containing 1% carboxymethyl cellulose (CMC) as the carbon source was dispensed in 250 ml Erlenmeyer flasks, then each inoculated with five mycelial discs (5 mm diameter) taken from the periphery of a growing colony on Vogel's CM. The flasks were incubated at 37 °C (stationary culture) for 8 days. The crude enzyme obtained by decanting the liquid was centrifuged at 22 000 g for 30 min at 4 °C. Cellulase was measured in terms of exoglucanase (FPase), endoglucanase (CMCase) and β -glucosidase [6].

Enzyme unit. Enzyme activity has been expressed in international units (IU) as the amount of enzyme needed to release one micromole of glucose (in case of exoglucanase and endoglucanase) or p-nitrophenol (for β -glucosidase) or their equivalent $\text{ml}^{-1} \text{min}^{-1}$.

Results

Mutants. In the present study 25 mutants of *Aspergillus nidulans* were used. The mutants could be distinguished morphologically on the basis of their spore colour. While most of the mutants were green, mutants with yellow, white and fawn coloured spores were also present. Thirteen mutants were found to have no auxotrophic requirement. Single nutritional requirement was observed for ten mutants whereas two mutants were found to be double auxotrophs (Table I).

Cellulase character. Mutants were grown for 14 days and a set of flasks was removed on alternate days for assaying the activities of exoglucanase, endoglucanase and β -glucosidase. Activity of wild type *A. nidulans* (ATCC 62041) on the eighth day was taken as control. Maximum activity for exoglucanase (0.073 IU/ml) and endoglucanase (0.26 IU/ml) was observed on the eighth day whereas β -glucosidase activity exhibited no decline till the termination of the experiment and the activity on the eighth day was 0.084 IU/ml. A range of variation was observed in the titre of the three enzyme components among the mutants. Repression or enhancement of the three enzymes in any given mutant was not correlated. Mutants exhibiting less than 30% of the wild type activity were taken as hypo-producers. As evident from Table I and Fig. 1, five mutants were found to be hypo-producers for endoglucanase, whereas six mutants had elevated titres as compared to the wild type. Endoglucanase activity ranged between 0.0–0.32 IU/ml. The range of titres for exoglucanase was found to vary between 0.0–0.087 IU/ml with three mutants showing enhancement and three having repressed activity. For β -glucosidase none of the mutants tested showed an increase over the wild type although seven mutants were found to be highly repressed. The range of activity for β -glucosidase was from 0.002–0.08 IU/ml. Among the mutants no correlation was detected between morphology, auxotrophy and cellulase character, however in most of the mutants, for endoglucanase and exoglucanase a definite correlation was observed between good growth (measured in terms of dry weight/flask) and cellulase production.

Table I

Comparative cellulase production by wild type and mutants of A. nidulans

Strain	Colour	Nutritional requirements	Enzyme activity (IU/ml)			Dry weight (mg/flask)
			exoglucanase	endoglucanase	β -glucosidase	
<i>A. nidulans</i> (w)	green	none	0.073	0.26	0.084	57
A-1	green	pyr	0.007	0.29	0.084	62
A-2	green	lys, pyr	0.02	0.06	0.075	35
A-3	green	nic	0.06	0.30	0.024	80
A-4	yellow	bio, tyr	0.051	0.13	0.002	50
A-5	yellow	pyr	0.05	0.07	0.012	30
A-6	white	none	0.087	0.28	0.025	58
A-7	green	none	0.051	0.14	0.025	40
A-8	green	none	0.051	0.04	0.005	25
R-21	yellow	paba	0.022	0.23	0.057	55
R-153	white	pyr	0.07	0.26	0.048	60
48 A	green	none	0.036	0.32	0.053	75
49 A	green	none	0.073	0.20	0.06	60
23 B	green	none	0.10	0.28	0.008	62
GO51	green	nic	0.073	0.25	0.02	57
1199	green	bio	0.0	0.146	0.036	50
1201	yellow-green	none	0.0	0.18	0.027	47
832	yellow	none	0.035	0.12	0.042	50
816	yellow	none	0.02	0.26	0.08	61
1209	yellow	cho	0.03	0.30	0.04	62
1212	fawn	none	0.0	0.087	0.027	30
1200	white	none	0.06	0.0	0.018	48
1213	green	ino	0.02	0.17	0.04	40
818	yellow	lys	0.018	0.02	0.002	45
1237	yellow	met	0.02	0.17	0.04	34
819	fawn	none	0.08	0.083	0.051	55

paba: p-amino benzoic acid; pyr: pyridoxine; lys: lysine; nic: nicotinamide; bio: biotin; tyr: tyrosine; met: methionine; ino: inositol

Endoglucanase and β -glucosidase production was also found to be related to development of conidia and cleistothecia.

Individual enzyme profiles of the mutants were studied, taking the profile of the wild type *A. nidulans* as control. As mentioned earlier, maximum production of exoglucanase and endoglucanase was attained on the eighth day which dropped gradually to 0.01 IU/ml and 0.09 IU/ml, respectively, by the fourteenth day. β -glucosidase exhibited a different pattern of profile. No decline was observed till the termination of the experiment. A yield of 0.11 IU/ml was assayed on the fourteenth day (Fig. 2). The

mutants exhibited variation as compared to the wild type. In some of the mutants a shift in the day of maximum production had taken place. In quite a number of mutants enzyme activity exhibited two peaks. There was no decline in endoglucanase activity even after 14 days of culturing in some cases. β -glucosidase, in most of the mutants followed a pattern similar to the wild type i.e. evincing no drop in activity till the termination of the experiment (Fig. 3a–y).

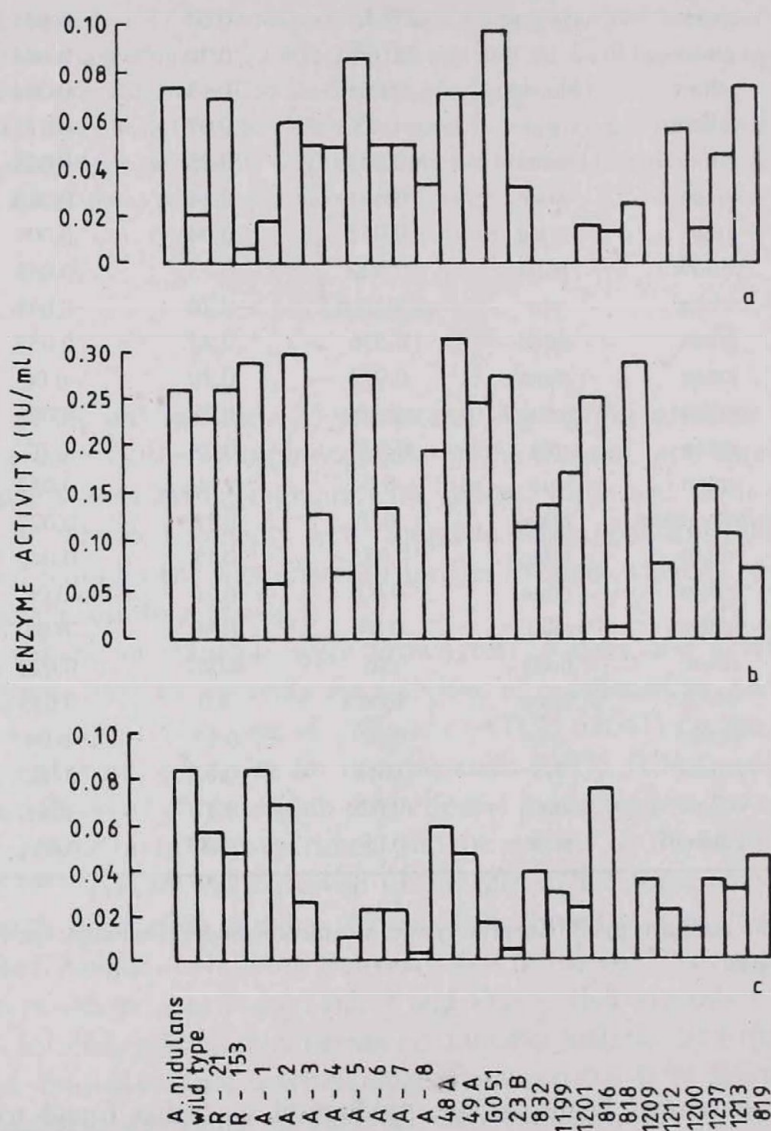


Fig. 1. Comparative cellulase activities of *A. nidulans* (wild type) and mutants (a) exoglucanase; (b) endoglucanase and (c) β -glucosidase

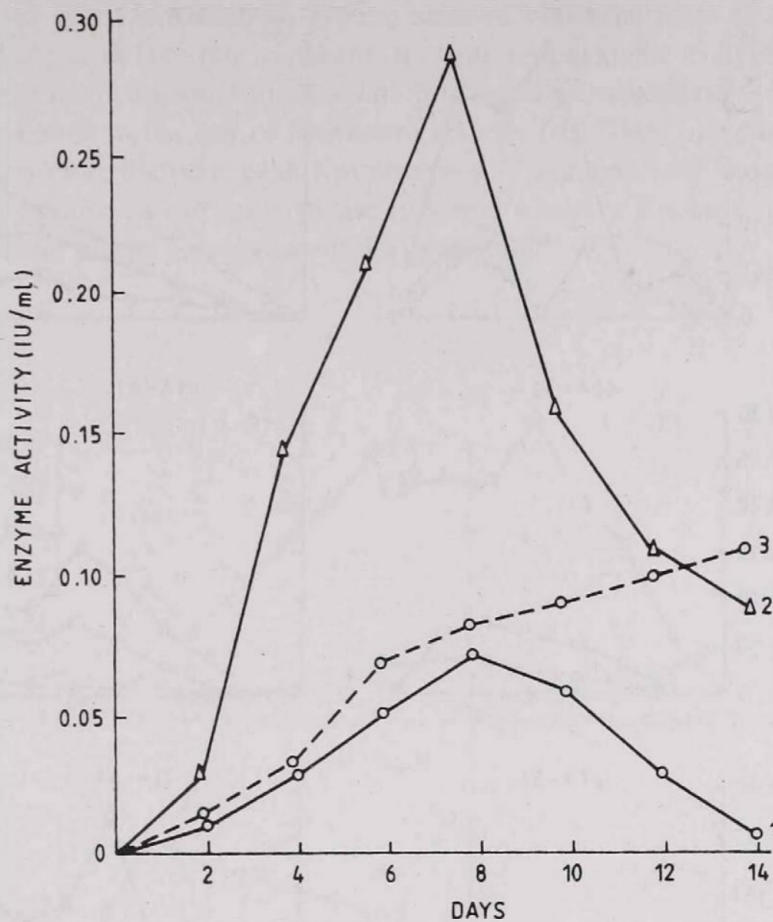


Fig. 2. Cellulase production by *A. nidulans* (wild type) exoglucanase (1); endoglucanase (2) and β -glucosidase (3)

Discussion

Considerable enhancement in the activity of various enzymes has been achieved through screening of the mutants. In many instances altered morphology and auxotrophy have been related to extracellular enzyme production. Junwei and Shuyun [7] reported variable cellulase producing ability by mutants differing with respect to spore colour in *Aspergillus niger*. In some cases correlation between growth and elevation in cellulase titre has been observed. Good and dark green conidiating faster growth with highly branched mycelia, growth in the form of compact colonies or tight pellets have also been associated with enhanced cellulase production in *Trichoderma longibrachiatum* [8] and various extracellular enzymes of *A. nidulans* [9]. The variability in the titres of the mutants could also be due to mutations affecting the permeability of the cell membrane which could allow a higher release of one enzyme and/or inhibit another one. As the

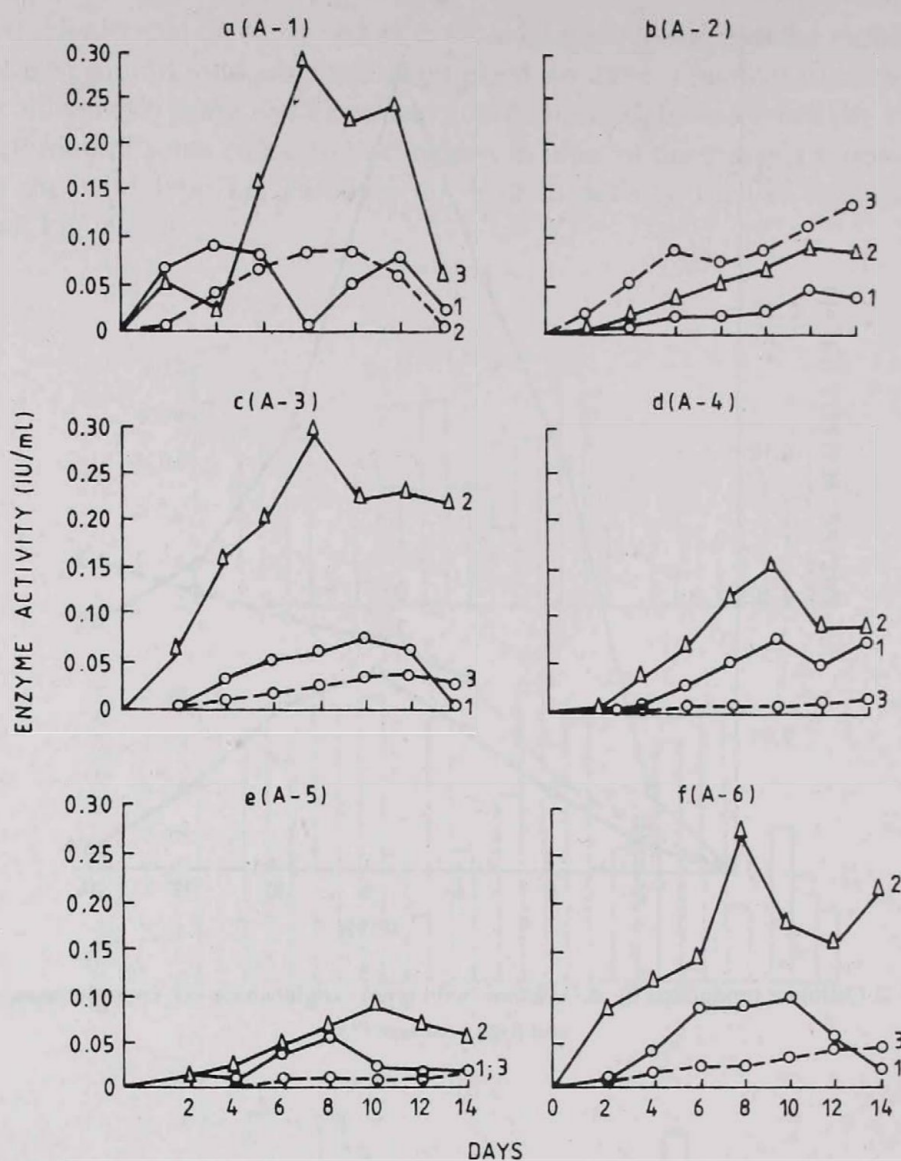


Fig. 3. Cellulase profiles of the mutants (a-f). Exoglucanase (1); endoglucanase (2) and β -glucosidase (3)

individual cellulases differ in their specificity and activity towards different cellulosic substrates, several enzyme preparations enriched with, or lacking a particular component could be useful, for instance for the optimization of processes.

Differential increase of the three components implies that the genes controlling the cellulase complex are not under coordinate control. Similar conclusions were reached in the instance of *T. reesei* mutants [10, 11] and in *A. nidulans* [12]. However, Ghosh et al. [5] are of the view that if all the three enzymes are enhanced, as in case of GO51, it could be that the mutation affects one component and the other enzyme of cellulase enzyme complex may increase due to coordinate regulatory mechanism. A wide range of hypercellulolytic mutants have been isolated. As a result of systematic improvement of production properties by mutagenesis and screening the best strains of *T. reesei* today

produce up to 40 g/l of extracellular protein most of which consists of cellulases [1]. A comparison of the cellulase profile of the mutants revealed the enzyme activity to be related to development. In some mutants the profile varied considerably as compared to the wild type. A shift in the day of maximum activity had taken place in some mutants, whereas in others more than one peak was observed. Variations were prominent in case of endoglucanase. In most cases β -glucosidase followed a pattern similar to the wild type i.e. evincing no decline till the termination of the experiment.

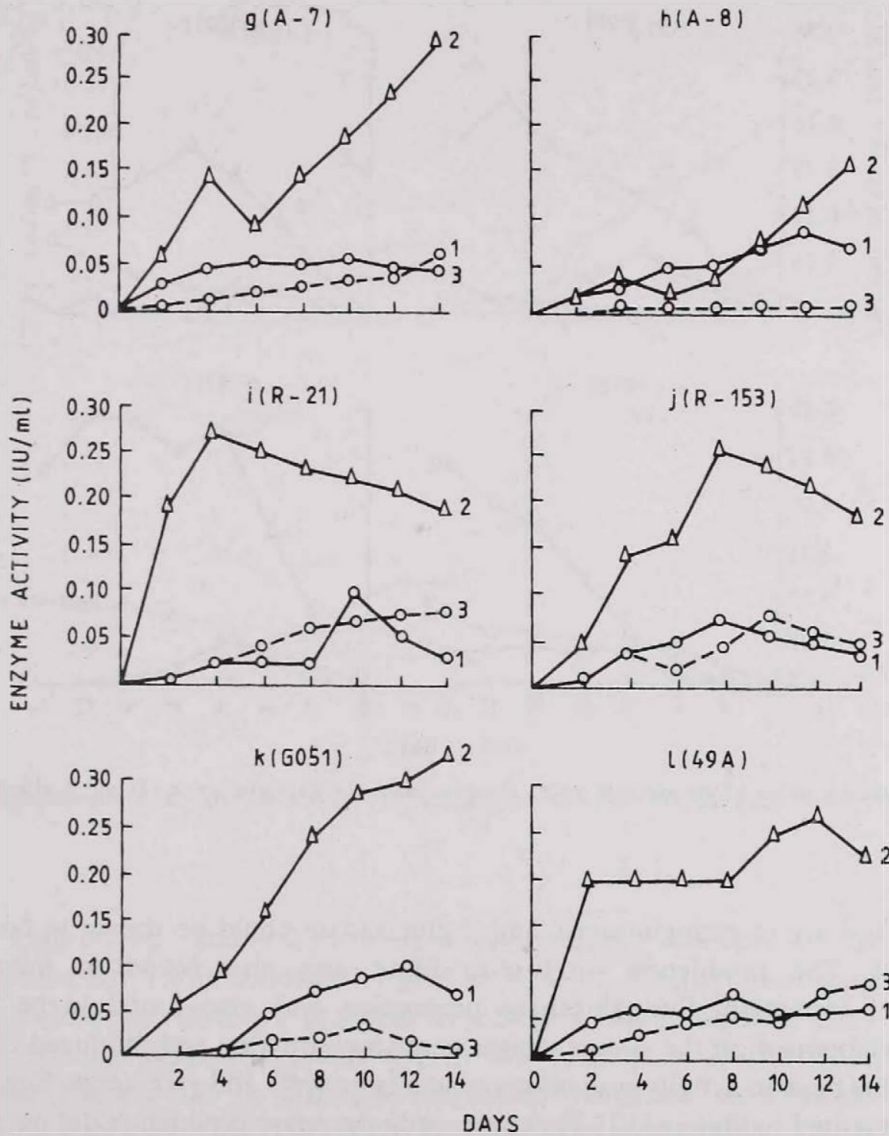


Fig. 3. Cellulase profiles of the mutants (g-l). Exoglucanase (1); endoglucanase (2) and β -glucosidase (3)

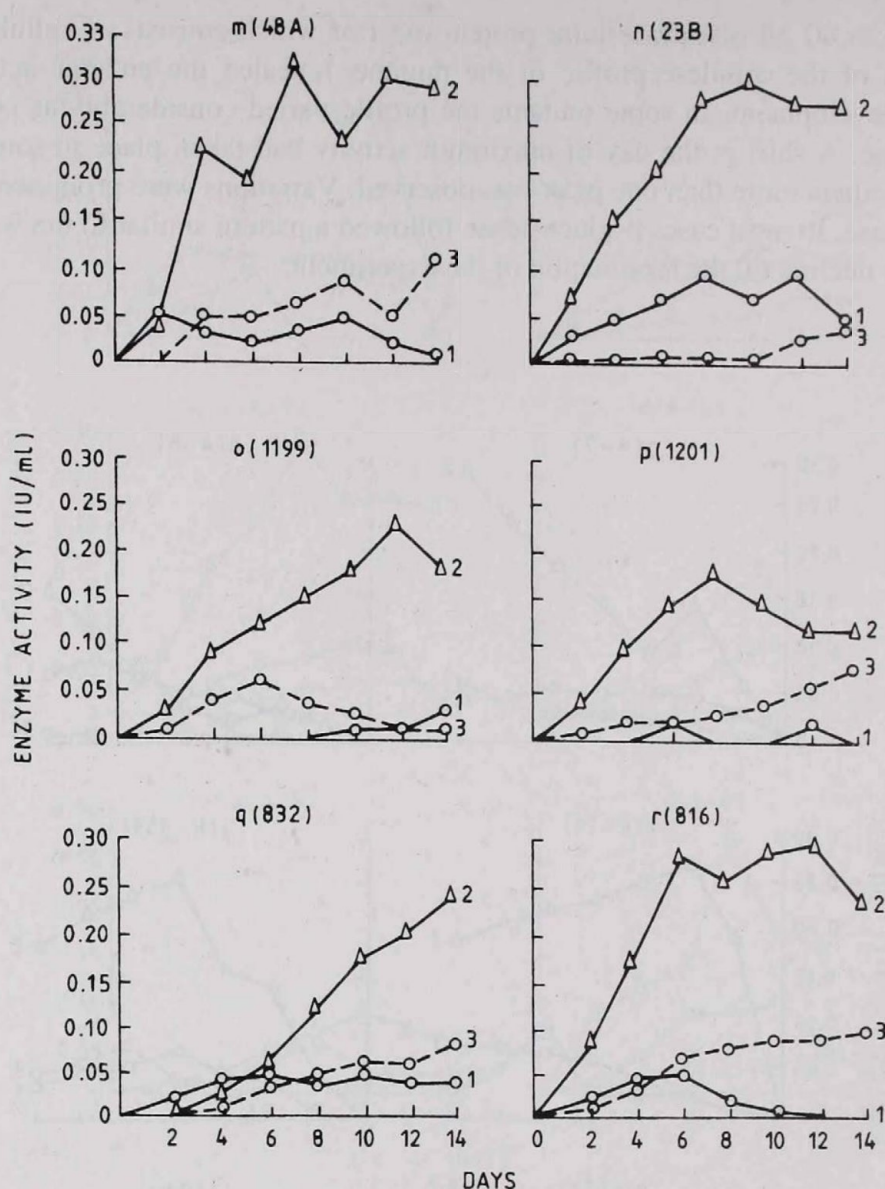


Fig. 3. Cellulase profiles of the mutants (m-r). Exoglucanase (1); endoglucanase (2) and β -glucosidase (3)

The activity of endoglucanase and β -glucosidase could be shown to be related to development. The production of β -glucosidase was also found to increase with cleistothecial formation. Endoglucanase production was also found to be related to conidial development. In the strains which were slow growing and produced conidia at a later stage, the peak in activity was observed at a later stage and vice versa. Similar results have been reported by Bagga [12]. However, endoglucanase production did not constantly increase as was the case with β -glucosidase. The decline in the production could be due to the degradation of the enzyme. The second peak could be formed as a result of release of intracellular endoglucanases due to cell lysis. In mutants (A-4; 1200) where the conidial as well as cleistothecial formation was low, enzyme activity was also repressed.

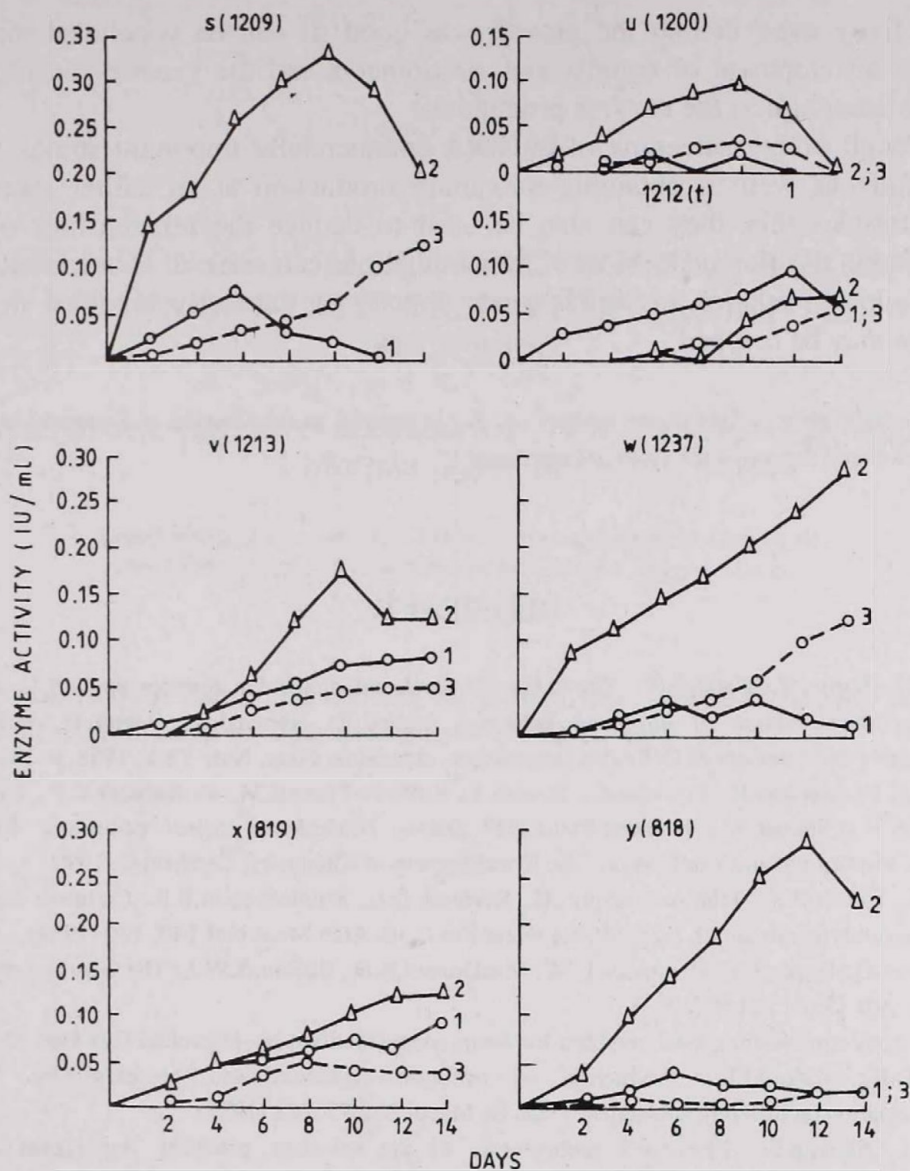


Fig. 3. Cellulase profiles of the mutants (s-y). Exoglucanase (1); endoglucanase (2) and β -glucosidase (3)

In case of R-21 and A-2 the exoglucanase activity was very low for nearly eight days of incubation, rising suddenly to give high yields. Similar observations were made for β -glucosidase of 23B. A probable explanation could be that these mutants are secretion deficient and the sudden rise in the activity is caused by release of the intracellular fraction due to cell lysis. Instances where the secretion of a product is increased by super secretor genes have also been reported Kotylak and El-Gewly [13]. The production of endoglucanase and β -glucosidase in most of the mutants could also be related to the growth. Dry weight of the mycelia (mg/flask) was determined. In mutants which exhibited good growth (49 A, A-3, 23 B, 1212, GO51, 48 A, 816, R-153, A-1) good enzyme activities were observed. However, besides good growth other parameters also affected the production of the enzymes. As mentioned above, A-4 and 1200 had low

enzyme activity even though the growth was good. It can be concluded that besides growth, the development of conidia and cleistothecia and the genetic constitution of a particular mutant affects the enzyme production.

Through proper screening of mutants, commercially important strains exhibiting improved titre as well as obtaining maximum production at an earlier stage, can be obtained. Besides this, they can also be used to deduce the relationships of enzyme production with development. Most of these mutations can serve to locate markers on the linkage groups in relation to which genes directly or indirectly involved in cellulose degradation may be mapped.

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ERRATUM

DETECTION OF BULLOUS PEMPHIGOID ANTIBODIES BY MEANS OF SYNTHETIC PEPTIDES AS ANTIGENIC EPITOPES

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Vol. 43, no. 4, p. 339, name and address of authors, line 4: József Molnár should read János Molnár; line 7: Géza K. Tóth should read Gábor K. Tóth

APPENDIX

REPORT OF THE COMMISSIONER OF THE GENERAL LAND OFFICE
ON THE PROGRESS OF THE SURVEY OF THE LANDS OF THE
UNITED STATES IN THE YEAR 1850

ALBANY: PUBLISHED BY J. B. LEECH, 1851.

THE COMMISSIONER OF THE GENERAL LAND OFFICE,
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THE LANDS OF THE UNITED STATES ARE
CLASSIFIED INTO SEVERAL CLASSES, AND
THEY ARE SURVEYED IN ACCORDANCE
WITH THE ACTS OF CONGRESS.

THE SURVEY OF THE LANDS OF THE
UNITED STATES IS A WORK OF GREAT
IMPORTANCE, AND IT IS THE DUTY
OF THE COMMISSIONER TO REPORT
ON THE PROGRESS OF THE SURVEY.

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Materials and methods. Describe microorganisms, methods, apparatus, procedures and statistical methods in sufficient detail to allow other authors to reproduce the result. This part may have subheadings like "Bacterial strains" or "Culture media".

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PROBLEMS OF MICROBIAL ZONOSSES IN HUNGARY

(A REVIEW)

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Abstract

Hungarian data of 36 zoonotic diseases are summarized. These illnesses cause problems not only for health, public health and veterinary health services but for the society and their importance has increased. Changing character of an old pathogen (*Salmonella enteritidis* PT4) resulted in new epidemiological situation. The number of cases of food-borne zoonotic diseases has rapidly grown.

The number of pets has elevated and in consequence the possibility of an infection of owners, breeders, sellers and other persons has also grown.

* This paper was partly presented on the Session of Microbial Zoonoses of the IUMS Congress 96, 8th International Congress of Bacterial and Applied Microbiol. Div., Jerusalem, Israel, August 18–23, 1996.

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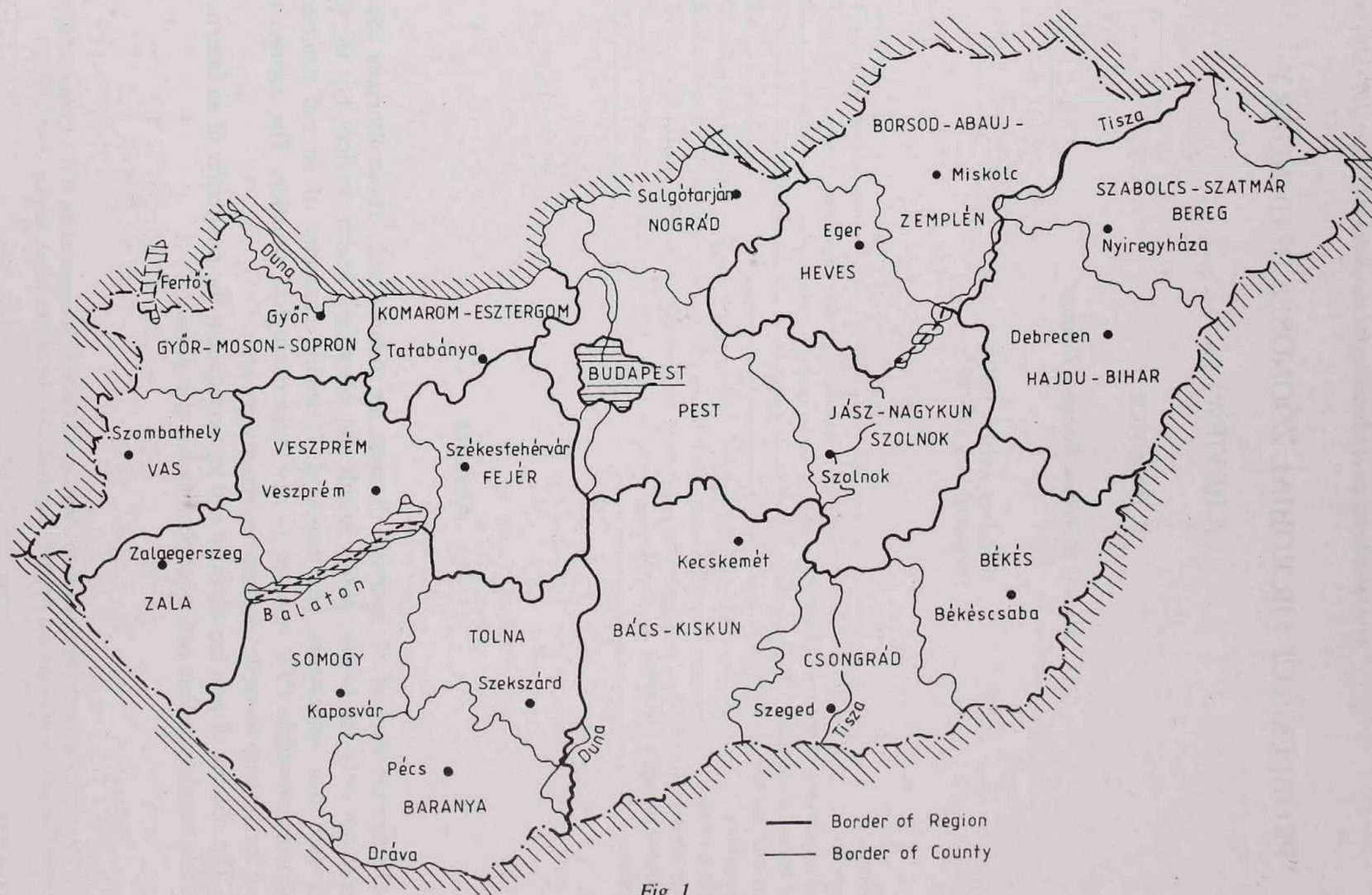


Fig. 1

Growing number of HIV positive patients as well as ill persons treated with immunosuppressive drugs increases the importance of opportunistic zoonotic pathogens (*Cryptosporidium*, *Toxoplasma*).

The most effective and less expensive way to prevent an exposed population is the active immunization (BCG, tetanus, tick-borne encephalitis). Active immunization of animals can also reduce probability of human infections and economic losses (leptospirosis, rabies).

In special cases it is advised to perform eradication programmes to get pathogenic-free domestic animals (brucellosis, bovine tuberculosis, salmonellosis, campylobacteriosis).

Permanent surveillance is obligatory to recognize changing nature of pathogens, alteration of epidemiological situation and to identify areas for further research. Continuous education of population in general and special teaching of risky groups are very important for an effective prevention.

Introduction

More than 150 zoonotic diseases representing every classes of infectious agents have been known all over the world. Climatic and geographical conditions essentially determine the presence of species of animal hosts, and kinds of pathogens as well as mode of their transmission from animals to humans. In addition to these factors socioeconomic conditions, public health regulations, living standard, educational level, nutritional, housing, working, husbandry and other habits of a population importantly influence occurrence of zoonotic diseases.

Hungary is a small Middle-European country (Fig. 1). Its territory is 94,000 km². The number of inhabitants is 10.2 million. The country is divided into 19 counties. Natural, geographical, economic and social conditions are different in these administrative units.

At present 51 infectious diseases are notifiable [1] (Table I). In addition to these laboratory data of 12 other infections have also been collected (Table II).

Using the list of World Health Organisation's (WHO) publication entitled "Bacterial and viral zoonoses" [2] as well as "Parasitic zoonoses" [3] at least 30 zoonotic diseases can regularly be diagnosed in our country. In addition, a few occasional infections and imported cases may also occur. Data of 36 zoonotic diseases which are collected either on obligatory or on voluntarily ways are presented and discussed in this paper.

Table I

Notifiable diseases in Hungary

Ancylostomiasis	Paratyphus
Anthrax	Parotitis epidemica
Brucellosis	Pertussis
Cholera	Plague
Diphtheria	Poliomyelitis
Dysentery	Q fever
EPEC (under 1 year)	Rubella (Rubeola)
Echinococcosis	Rubella congen. syndr.
Encephalitis infec. (Tick-borne)	Salmonellosis
Febris flava (Yellow fever)	Scarlet fever
Febris recurrens (Relapsing fev.)	Schistosomiasis
Glanders	<i>S. aureus</i> inf. (<1 y age)
German measles (Rubella)	Taeniasis
Hepatitis infectiosa	Tetanus
Keratoconjunctivitis (epidemic)	Toxoplasmosis
Leprosy	Trichinosis
Leptospirosis	Tularemia
Lyssa (Rabies)	Typhus abdominalis
Malaria	Typhus epidemic
Meningitis epidemica	Variola
Meningitis serosa (aseptic)	Wund susp. rabies
Mononucleosis infectiosa	
Ornithosis (Psittacosis)	AIDS
	Chancroid
Tuberculosis	Gonorrhea
	Granuloma inguinale
	Lymphogranuloma vener.
	Syphilis

General importance of zoonotic infections

Summarized morbidity and mortality data of zoonotic diseases are compared with those of notified infectious illnesses in Table III.

The data of Table III show that between 1990 and 1994 229,991 cases of 38 infectious diseases were notified in Hungary. Influenza, tuberculosis and classic STD cases are not included in this figure. 373 deaths were registered in consequence of these diseases. 79,621 out of the 229,991 cases were zoonotic diseases (34.6%). 164 patients died from the 79,621 cases, so the lethality was 0.25%. The lethality of the zoonotic

diseases was higher than that of non-zoonotic illnesses. It is evident that zoonotic diseases result in problems not only for health, public health and veterinary health services but for the total society.

Table II

List of infectious diseases voluntarily reported in Hungary

Amebiasis
Campylobacteriosis
Cryptosporidiosis
Dermatophytosis
EIEC infection
EHEC infection
Giardiasis
Haemorrhagic fever with renal syndrome
Listeriosis
Tick-borne encephalitis
Toxocariasis
Yersiniosis

Table III

Notified cases of 38 infectious diseases and number of deaths of infectious diseases in Hungary 1990–1994

Disease	Number of	
	cases (in %)	death (in %)
18 zoonotic diseases	79,621 (34.6)	164 (43.9) (lethality 0.25)
20 non zoonotic diseases	150,370 (65.4)	209 (56.1) (lethality 0.13)
Total 38 infectious diseases (a)	229,991 (100)	373 (100) (lethality 0.16)

(a) except influenza, tuberculosis and classic STD-s. Imported cases are included

Table IV

Bacterial zoonoses cases in Hungary, 1972–1994

Disease (Order of notification was issued)	Number of cases in years			Total number of cases
	1972–81	1982–91	1992–94	
Anthrax (1930)a	28	4	2	34
Borreliosis (–)	The first case was in 1984			unk.
Botulism (1960)d	26	157	36	219
Brucellosis (1949)	797	75	4	876
Campylobacteriosis (–)	unk.	49975	28759	78734
<i>C. perfringens</i> fbd. (1960)d	3102	3632	444	7178
Glander (1930)a	0	0	0	0
Leptospirosis (1958)	547	397	109	1053
Listeriosis (–)	24	99	63	186
Ornithosis (1966)	230	36	4	270
Plague (1930)a	0	0	0	0
Q fever (1966)	78	61	10	149
Salmonellosis (1958)b	55848	112970	69064	237882
<i>S. aureus</i> etox. (1960)d	unk.	3126	476	3602
Tetanus (1930)a	594	283	54	31
Tuberculosis bp. (1930)a	49196	28006	5766	82968
<i>M. bovis</i> positive cases	32	19	5	56
Tularemia (1949)	411	489	201	1101
<i>Yersinia</i> pfaeces (–)	3714c	5664	937	10315

Occasional diseases: Cat scratch disease, Erysipelas, Erysipeloid, Group B Streptococcus infection, *P. multocida* infection, Staphylococcal infection, *Y. pseudotuberculosis* infection

unk. = unknown

fbd. = food-borne disease

etox. = enterotoxigenic

bp. = bacteriologically positive

pfaeces = positive faeces

a = These diseases were ordered to be notified in 1876

b = Paratyphus and food-borne cases have been notified since 1930

c = Data of two years are missing

d = Data of food-borne diseases are collected in the National Institute of Food Hygiene and Nutrition [8]

Data of bacterial zoonoses

Data of bacterial zoonoses are presented in Table IV. Infections caused by *Salmonella* and *Campylobacter* strains proved to be dominant. They are followed by *Yersinia* infections, staphylococcal enterotoxigenic and *C. perfringens* food-borne

diseases. These enteric illnesses are most frequently transmitted by foods. Importance of single enteric pathogens can be judged on the basis of Fig. 2. *Salmonella* strains occur most frequently. *Campylobacter*, *Shigellae*, *E. coli* and *Yersinia* strains are also important [4]. In the USA *Salmonella* species are at the second place behind *Campylobacter* causing an estimated 2 million diseases annually [5].

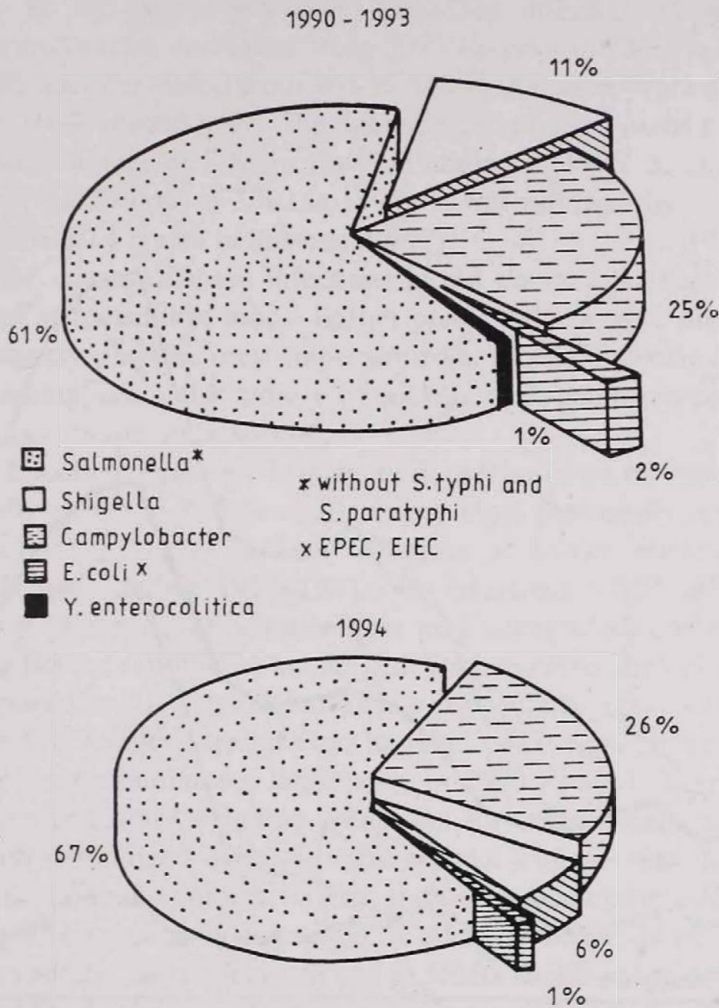


Fig. 2. Frequency distribution of enteric pathogens in patients and carriers by year in Hungary, 1990-1994

Figure 3 shows the number of salmonellosis cases and that of *Salmonella* strains. Since the end of the seventies, with the exception of four short periods, both the number of ill persons as well as that of isolated strains have continuously increased. The age specific morbidity rate is the highest under 5 and over 50 years. Lethal cases occurred in these age groups. Up to 1979 *S. typhimurium* strains were dominant but since that time *S.*

enteritidis strains have exceeded them. Now more than 80% of all *Salmonella* strains are *S. enteritidis*. Greater part of these *S. enteritidis* strains were PT4. They can be found inside hatching eggs.

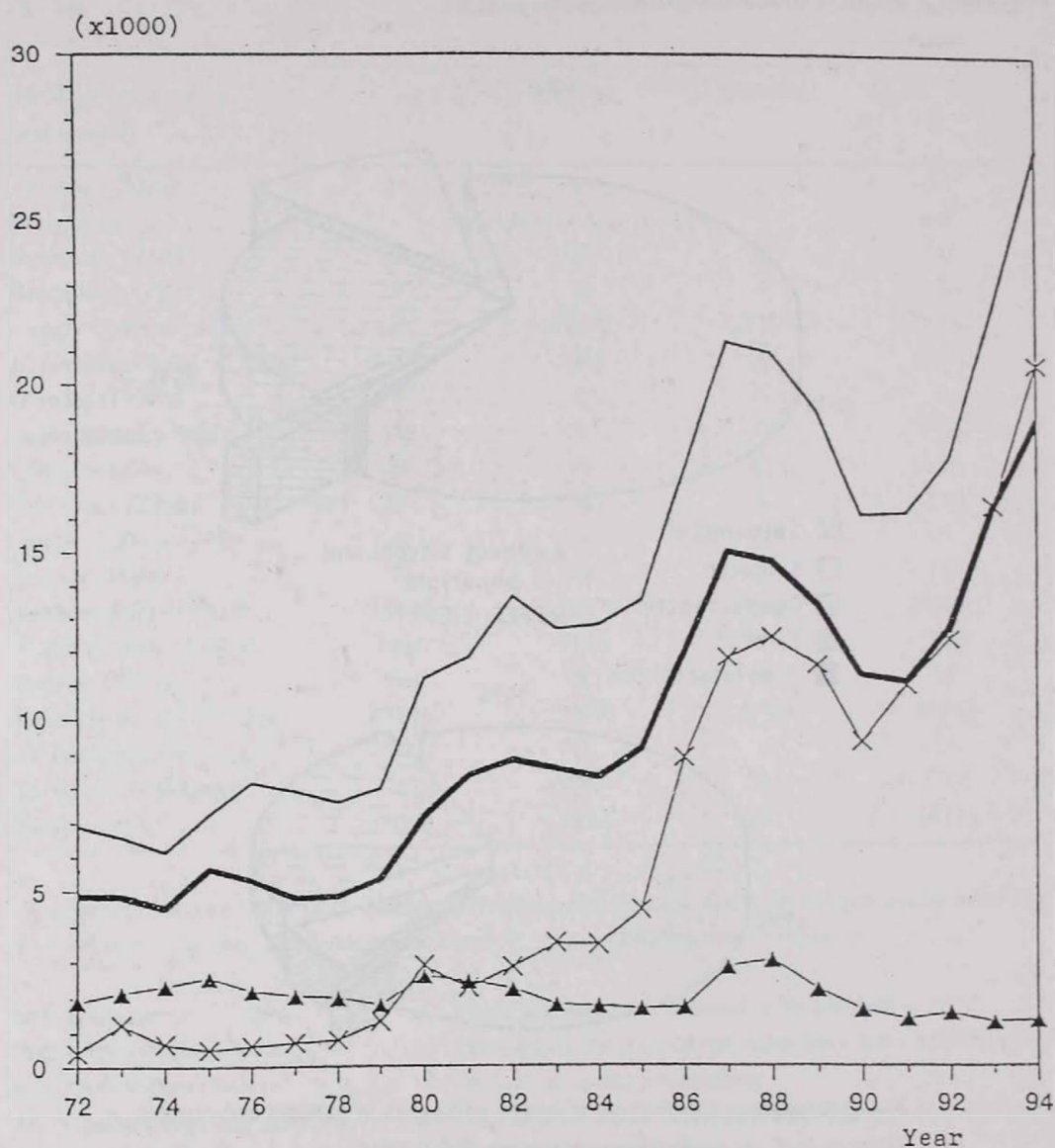


Fig. 3. *Salmonella* strains and reported salmonellosis cases by year in Hungary; Number of salmonellosis cases (—), number of all *Salmonella* strains (—), number of *S. typhimurium* strains (—▲—▲—), number of *S. enteritidis* strains (—x—x—)

This *Salmonella* caused the serious epidemic situation in the U.K. during the eighties [6]. In the United States among the more than 2000 *Salmonella* serotypes *S. enteritidis* has ranked first or second in frequency of isolation from humans since 1988 and accounted for 21% of reported isolates in 1993. Each year an average of 55 outbreaks of *S. enteritidis* infections were reported to Centers for Disease Control and Prevention (CDC). Approximately 11% of patients were hospitalized and 0.3% died [5].

In contrast to the above-mentioned data the number of reported human salmonellosis cases in Sweden decreased from 5097 in 1994 to 3562 in 1995. The total number of domestic cases decreased from 865 to 558 in the same years and the imported cases from 4232 to 3004, respectively. The decrease in imported cases can be explained by the fact that fewer number of Swedes traveled abroad in 1995. *S. enteritidis* was the most frequent serotype (1579 out of 3562 in 1995). It was followed by *S. typhimurium* in 452 cases. *S. enteritidis* PT4 is rare in this country [7].

Examining the causes of these infections in our country marked changes can be observed in the kind of transmitter foods. Earlier pork products were predominant. Now egg products and poultry play the most important role. Most frequently insufficient heat treatment during cooking, unsuitable storage of cooked foods and cross-contamination are the causes of these food-borne outbreaks [8].

Food-borne infections cause an estimated 6.5 million cases of human illnesses and 9000 deaths annually in the U.S. *Salmonella* is the most commonly reported cause of these events, accounting for 28% of such outbreaks of known etiology and 45% of outbreak-associated cases during 1973–1987. An estimated 0.01% of all shell eggs contain *S. enteritidis*. Outbreaks of salmonellosis may occur when commercial kitchens and those of private houses serve foods made with contaminated shell eggs that have not been sufficiently cooked to kill *Salmonella*. This is particularly likely when refrigeration is inadequate or holding temperatures are too low and when eggs are pooled whereby a single infected egg can contaminate a large pool. In 1990 Federal Drug Administration (FDA) issued recommendations including guidelines for refrigeration, cooking, pooling and substitution with pasteurized eggs. As most serious illnesses and deaths associated with salmonellosis occur among infants, elderly and immunocompromised persons, foods for these groups should be especially controlled. In addition, hospitals, nursing homes and commercial kitchens should use pasteurized egg products for all recipes requiring pooled eggs or lightly cooked eggs and should refrigerate all eggs and egg products [9].

Campylobacter germs are the second most frequent enteric pathogenic bacteria (Fig. 4). Incidence rate of campylobacteriosis cases has permanently increased. Campylobacteriosis is a typical illness of childhood. The age specific morbidity rate is the highest under 1 year [4]. Contaminated food, lack of suitable kitchen technics and knowledge may play a role in transportation of *Campylobacter*.

This explanation is valid in cases of yersiniosis, *C. perfringens* food-borne illnesses, *S. aureus* enterotoxigenesis and listeriosis cases, too. The number of *Yersinia* positive persons, *Staphylococcus* enterotoxigenesis cases and *C. perfringens* food-borne diseases has decreased. In contrast with these the number of listeriosis cases has increased. Neither epidemic listeriosis outbreak nor *Listeria* transmitter role of a food have been verified yet [10]. Botulism is connected in almost all cases with home made

pork products, smoked ham and smoked sausages after home slaughtering. The number of botulism cases increased. In all cases except one "B" type of botulotoxin was the causative agent.

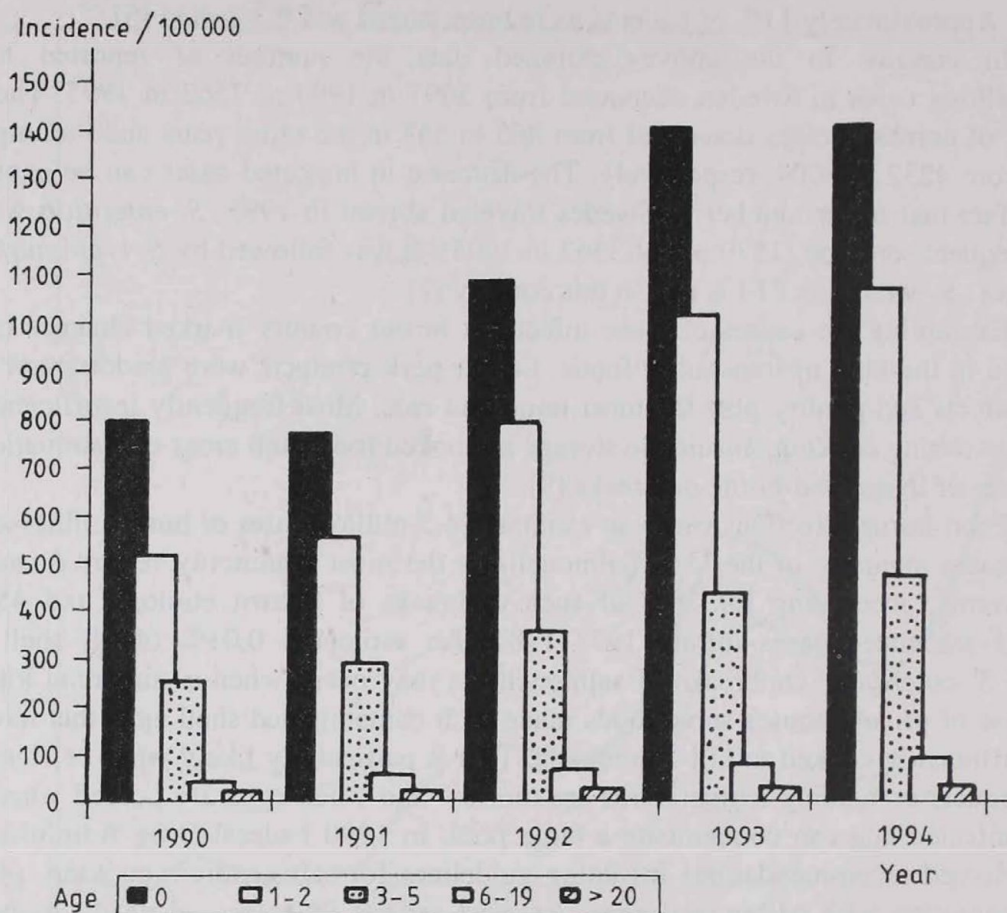


Fig. 4. Incidence rate of Campylobacter positive cases by age and year in Hungary

Occupational bacterial infections

Occupational diseases have to be reported to the Public Health Service in our country [11]. Next those notified bacterial infectious diseases will be discussed which may be occupational zoonoses, too. In consequence of veterinary regulations and eradication programmes *B. abortus* infected cows and *B. suis* infected pigs till 1985 and *M. bovis* positive oxen until the end of the eighties disappeared from our country [12]. The result of these favourable changes can be recognized in decreasing of the number of human brucellosis cases and in that of the number of *M. bovis* strains isolated from

human samples. In spite of this convenient epidemiological situation *B. suis* may be naturally harboured by wild hare. In the United States human brucellosis has been a reportable disease since 1947. In 1992, 105 cases were reported to CDC compared with a peak of approximately 6300 in 1947. Because of the variable clinical manifestations of brucellosis, only an estimated 4–10% of cases are recognized and reported. A unified national program to eradicate swine brucellosis was initiated in 1961. On December 31, 1993, 34 swine herds nationwide were under quarantine for brucellosis in seven states [13].

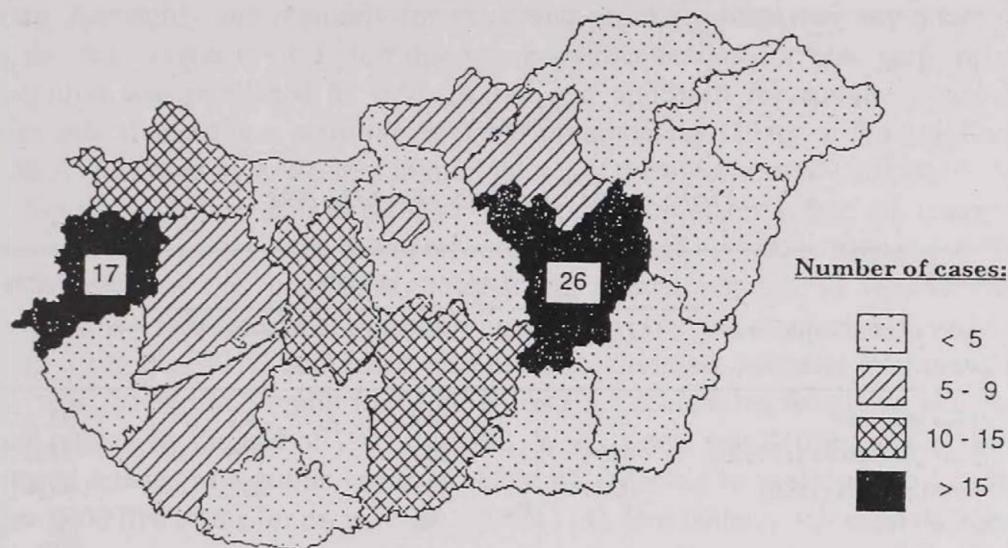


Fig. 5. Reported tularemia cases by county, in Hungary

During the past years human anthrax become rare. In contrast with this the number of tularemia cases has increased. Two distinct endemic foci have existed (Fig. 5). One of them is the West-Hungarian Vas county and its surroundings where hares carry *F. tularensis*. Hunters and their household members got the infection during winter months. The other one is on the Great Plain. Hamsters are the carriers there. These animals are caught for their fur in summer. So seasonality of Francisella infection of hamster hunters exists in this period. Mainly hurts and wounds on hands are the gates for the microbe in both types of infections [14].

Q-fever, ornithosis and one part of leptospirosis cases are occupational zoonotic diseases, too. *C. burnetii* infects mainly breeders of cows, shepherds and veterinaries because oxen and sheep may carry this bacterium. *Chlamydia psittaci* is mainly carried by ducks, geese as well as turkeys and in consequence of this fact those employees got ornithosis who breed or slaughter these birds.

Different types of leptospirae – *L. sejroe*, *L. pomona*, *L. icterohaemorrhagiae*, *L. grippityphosa*, *L. australis*, *L. hardjo*, *L. canicola* and *L. tarassovi* – can be found in oxen, pigs, dogs, rats and other rodents. These animals can shed leptospirae in their excreta,

mainly in urine [12]. Human beings can be infected directly with the excreta of a carrier or indirectly during bathing or working in a contaminated surface water. Leptospirosis has a characteristic seasonality. 60–70% of the cases are diagnosed from May till September. Farmers, animal keepers as well as breeders, slaughterhouse and food industrial employees, laboratory workers, miners and sewage men may be exposed. *L. sejroe*, *L. pomona* and *L. icterohaemorrhagiae* serotypes infect most frequently humans but *L. grippityphosa*, *L. australis* and other leptospirae can also be diagnosed as causative agents. Immunization of animals (oxen, pigs and dogs) is not only a preventive action for humans decreasing the number of infectious foci but vaccination can minimize economic losses, too.

Table V

Viral zoonoses cases in Hungary, 1972–1994

Disease (Order of notification was issued)	No. of cases in years			Total number of cases
	1972–81	1982–91	1992–94	
HFRS sp. (–)a	unk.	unk.	45	45
Meningitis serosa (1949)b	4194	2647	439	7280
Mononucleosis (1966)	5233	8317	2855	16404
Rabies (1930)	1	3c	2	6c
Encephalitis epidemica (1930)d	3197	3907	1009	8113
Wunds suspected for rabies (1930)	18760	26226	16663	61649
Yellow fever (1930)	0	0	0	0

Occasional diseases: A few Milkera nodes (Parapoxvirus infection) were diagnosed

HFRS = Haemorrhagic fever with renal syndrome

sp. = serologically positive

unk. = unknown

a = The first case was clinically diagnosed in 1953

b = LCM and other viral or bacterial infections are included

c = Imported cases are included

d = Since 1977 tick-borne encephalitis cases have been identified

The first Lyme borreliosis was diagnosed in 1984 in our country [15]. *Ixodes ricinus* is the vector for *B. burgdorferi*. It can be supposed that about 10% of ticks carry *Borrelia*. Mice are the main reservoirs. This is a disease of place; human beings are exposed when enter the territory of infected ticks either by occupational or recreational purpose. It is necessary to know where are these dangerous places. Early diagnosis of

Lyme borreliosis is important for effective antibiotic treatment and for the total recovery. It can be diagnosed by the earliest manifestation that is by so-called bull's eye type erythematous rash ≥ 5 cm in diameter (erythema chronicum migrans). The number of persons infected annually is not known.

After an 8 years long surveillance programme Lyme disease has been a notifiable disease since 1990 in the U.S. In 1994, 13083 cases were reported, 58% more than the 8257 cases reported in 1993. The overall incidence of reported Lyme disease was 5.2 per 100,000 population. This disease is the most commonly reported vectorborne infectious disease in the U.S. The risk for infection can be reduced through avoidance of known tick habitats, other preventive measures include wearing long pants, applying tick repellents, checking thoroughly and regularly for ticks, and promptly removing any attached ticks. As to the serodiagnosis of Lyme disease recommendations for test performance and interpretation was published in 1995. A two-test approach for active disease and for previous infection using a sensitive enzyme immunoassay (EIA) or immunofluorescent assay (IFA) followed by a Western immunoblot was the algorithm of choice [16–18].

Since the fifties well organized, systematic, obligatory, free of charge yearly immunization campaigns have been performed to prevent infectious diseases in Hungary. In consequence of this continuous programme annual number of tetanus cases has decreased. It is a rare disease of persons who are over 55 years of age.

In 1989 the WHO adopted a resolution to eliminate neonatal tetanus worldwide and in 1990, the World Summit for Children issued a declaration for global elimination of neonatal tetanus by the end of 1995. In 1993, WHO's goal was defined as the elimination of neonatal tetanus as a public health problem by reducing its incidence to less than one case per 1000 live births for each health district [19]. Our country achieved this goal years before.

Since 1986, two human tick-borne diseases caused by *Erlischia* spp. have been recognized in the U.S.: human monocytic erlichiosis and human granulocytic erlichiosis. Since 1994 serum specimens from potential cases were tested for antibodies to *Erlischia* spp. and/or the presence of DNA of *Erlischia* agent by PCR assay. *Erlischia* agents were identified in ticks of deer and dog. Persons spending time outdoors in tick-infested areas should take precautions [18, 20]. In Hungary *Erlischia* infections were observed among animals but human infection has not been verified yet [12].

Viral zoonoses

As to viral zoonoses 5 diseases and the wounds suspected for rabies are notifiable (Table V). The present epidemic of rabies started in 1954. The virus arrived from Czechoslovakia and Russia and spread through the country. Foxes are the transmitters. Between 1983–1992 11035 rabid animals were diagnosed (Table VI). 84.1% out of the all were foxes, 1.5% other wild animals (deer, wild cat, rat), 6.6% cats, 3.8% dogs, 3.0% oxen and 0.74% other domestic animals [12]. Yearly immunization of dogs is obligatory long ago, therefore their transmitter role is limited. In spite of this fact 75% of persons were immunized because they were wounded or contaminated by suspected dog or cat

and only 25% of them were immunized because they touched a fox. In 1994 two persons died in rabies. Cat which later on proved to be rabid hurted them. The G.P. did not suspect rabies and did not immunize them. Since that point of time both the number of persons suspected for infection by rabies virus and the consequent immunizations – postexposure prophylaxis (PEP) – are high. In 1994 7022 persons were immunized and in 1995 6915 PEP were performed [21].

As to the future near the Austrian border immunization of foxes with attenuated living vaccine in lure set out by aeroplane has started in 1992 and this activity has spread in East direction. In 1996, the total Transdanubial territory was covered by lure. It is planned to eliminate fox rabies on that way. Preliminary results of the immunization are promising [22] (Fig. 6).

Table VI

Number of rabid animals by species in Hungary 1983–1992

Species	Number	% in total
Fox	9285	84.1
Other wild animals	175	1.5
Cat	735	6.6
Dog	423	3.8
Oxen	334	3.0
Sheep, goat	50	0.45
Pig	25	0.22
Horse, donkey	8	0.07
Total	11035	99.74

Since the 1950s, cases of human rabies in the U.S. have steadily declined. During 1980–1995, only 28 cases occurred and no human deaths were attributed to the racoon rabies virus variant associated with the epizootic which has spread from a focus in West Virginia since 1977. During 1993, nearly 6000 racoons were confirmed with rabies in this region. The rabies virus circulates among racoons, skunks, coyotes, foxes and bats. It is very important to ensure vaccination of all dogs and cats and to remove strays and unwanted animals. This programme has reduced laboratory-confirmed rabies cases in dogs from 6949 in 1947 to 153 in 1994. Because more rabies cases are reported annually involving cats than dogs, vaccination of cats should be required. During 1995, a total of four cases of human rabies were documented. In all four cases the rabies virus variant was associated with insectivorous bats. Importance of bat-related virus variants has increased in the epidemiology of human rabies. One of the national health objectives for the year 2000 is to reduce the number of rabies PEP administrations to no more than 9000 per year because of the associated costs and potential risks for adverse reactions. The cost of programmes to prevent rabies have increased in parallel with the spread of the epidemic in

consequence of the increase of the number of persons receiving PEP. Rabies control in wildlife through oral vaccination is being evaluated in the U.S. In 1992, a programme to administer vaccinia-rabies glycoprotein recombinant vaccine orally to racoons was initiated in Cape May County. Similar programmes are being planned for coyotes and foxes in other places. Population reduction of wildlife rabies reservoirs is not a recommended or cost-effective method for rabies control in the U.S. [23-27].

Tick-borne encephalitis is known long ago as Frühsommer- or Middle European meningo-encephalitis. Rodents, small mammals, wild animals, birds and occasionally domestic animals (oxen, goat, sheep) may be reservoirs of the virus. The pathogen is transmitted by ticks. Human beings are accidentally infected by tick entering its ecosystem. Raw milk of infected domestic animals can also transmit the virus to consumers. You can see the incidence of tick-borne encephalitis on the map [28] (Fig. 7). The Western and the Middle-North Hungarian territories belong to the big Middle European (Austrian, Slovenian, Slovakian and Croatian) focus. Seasonality of tick-borne encephalitis cases is characteristic and strongly connected with the activity of ticks. 70% of them are diagnosed in May, June and July and 10% in October [14]. Tick-borne encephalitis is a typical occupational and recreational disease. Specific and non-specific possibilities exist for the prevention. The first is active immunization of risky persons and domestic animals. The non-specific possibilities are suitable clothing, body control for tick and education. In our country the number of cases decreases.

Haemorrhagic fever with renal syndrome was first clinically diagnosed in 1953 as nephrosonephritis haemorrhagica [29]. Since that time some hundred cases were observed and some part of them were verified with serological tests. Small rodents (*Apodemus agrarius*, *Apodemus flavicollis* and voles) are the reservoirs of Hantaviruses. Three distinct antigenic structures, Hantaan, Puumala and a non-identified one were observed. Two separate endemic foci exist. One is the South-West Hungarian region near Slovenia, Croatia and Austria and the other is the North-East Hungarian region which is connected with the Slovakian focus (Fig. 8). The number of cases in Budapest is higher than in the reality, as patients are transported to there from the counties and the true origin of their infection is not always registered. Mainly workers of agriculture and forestry as well as military persons were infected. Seasonality with two peaks can be seen. The first one is in June, July and August, the other in September, October and November. To protect people it is necessary to educate them and to kill rodents, if this action seems to be effective [30, 31].

In 1993, a new hantavirus-associated pulmonary disease was recognized in the U.S. Rodents are the reservoir of the new virus, which does not cause apparent illness in its host. Infected rodents shed virus in saliva, urine and faeces for many weeks. Human infection may occur by inhalation of aerosols, by contamination of wound or conjunctiva, and possibly by ingestion of contaminated food or water [32].

Meningitis serosa cases have been notified since 1949. First all non-purulent bacterial and viral illnesses were registered under this nomination. Since 1958 *Leptospira* meningitis cases have been separated from meningitis serosa. Since 1977 meningitis cases caused by Flaviviruses have been classified into tick-borne encephalitis. At present LCM illnesses and other serologically non-identified viral and bacterial infections are represented by the number of meningitis serosa cases.

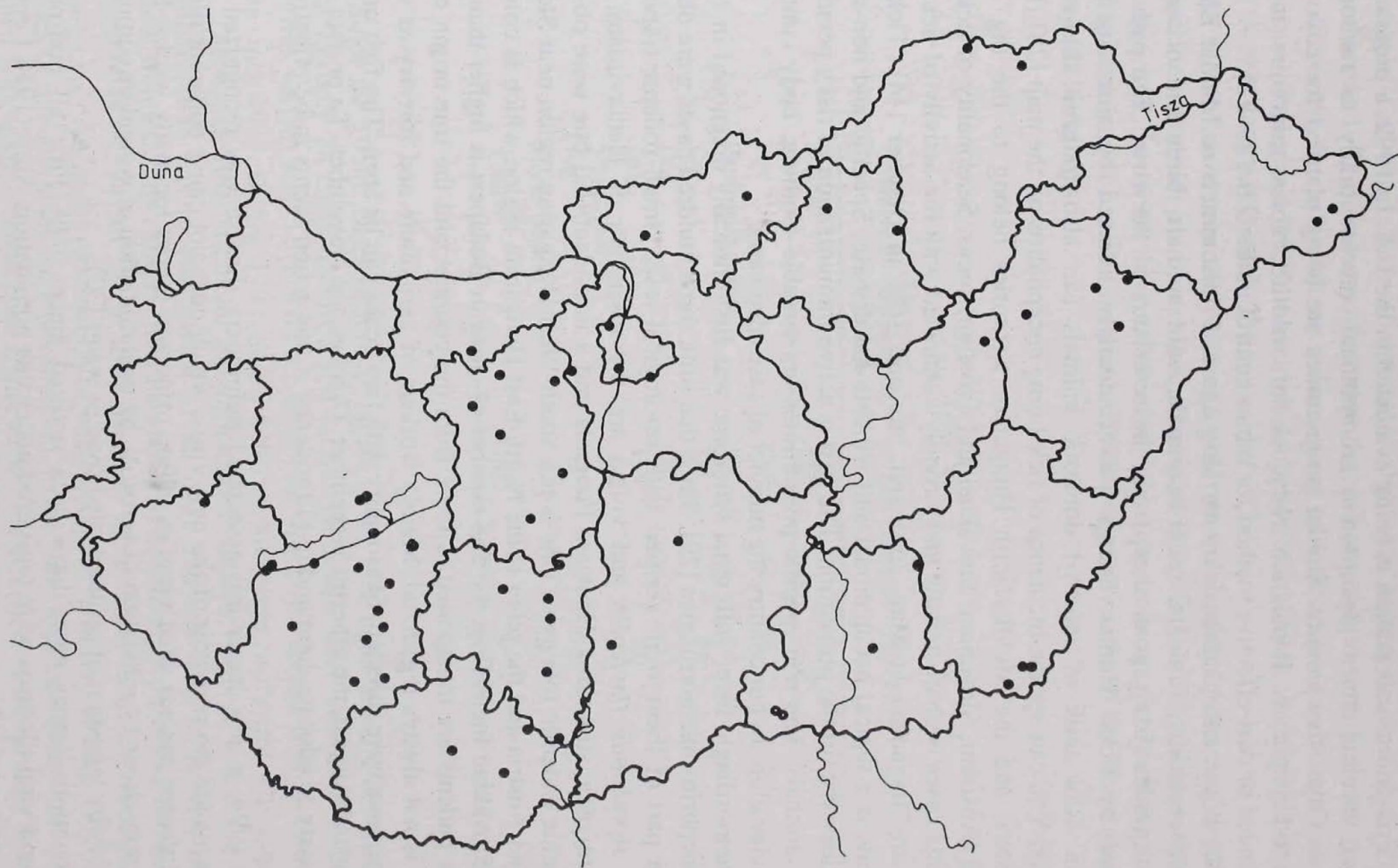


Fig. 6. Animal rabies cases by county in Hungary in June, 1996. Gray colour shows territory where active immunization has been performed

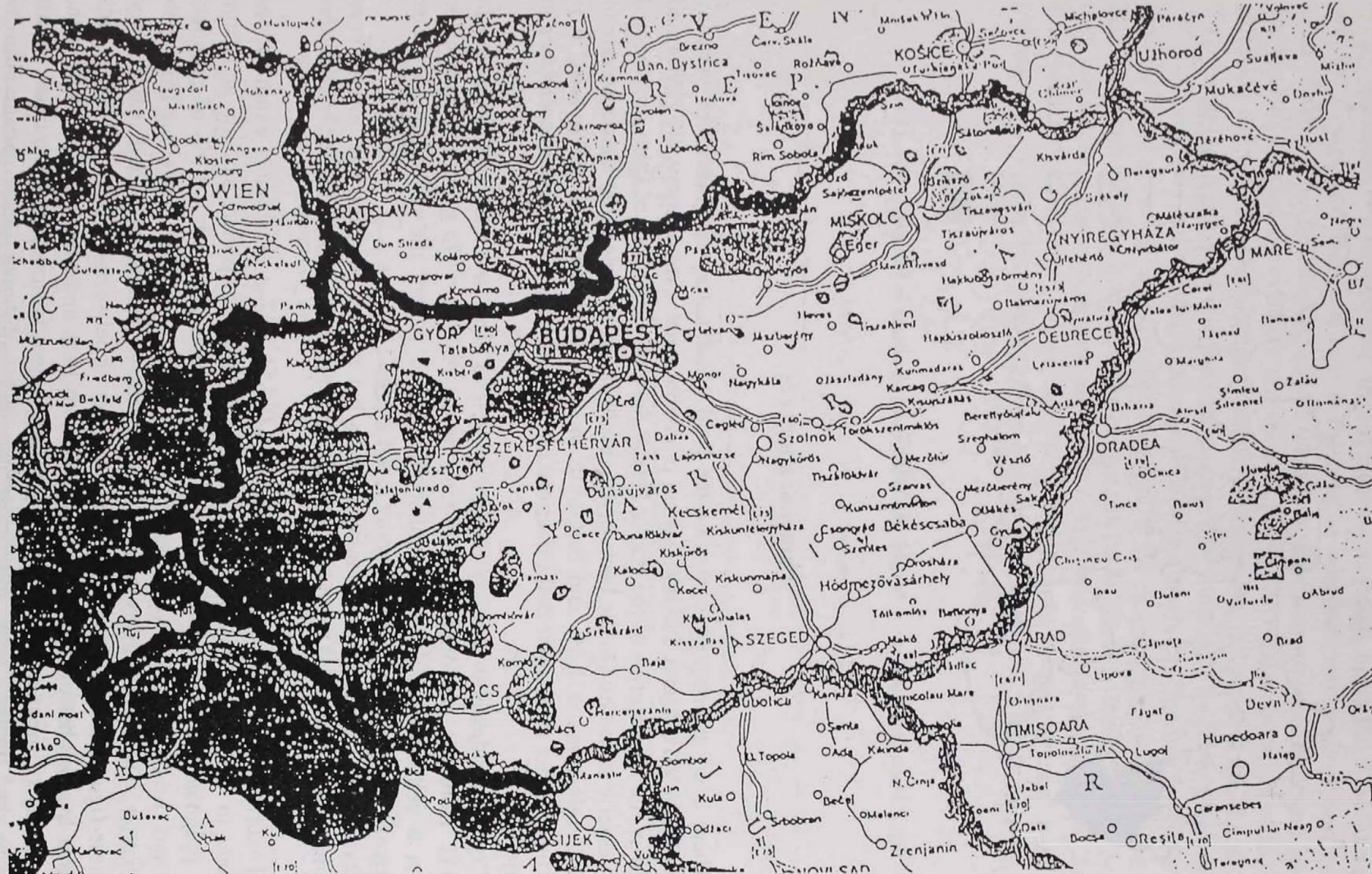


Fig. 7. Incidence of tick-borne encephalitis in Middle Europe. The darker spot means higher incidence rate

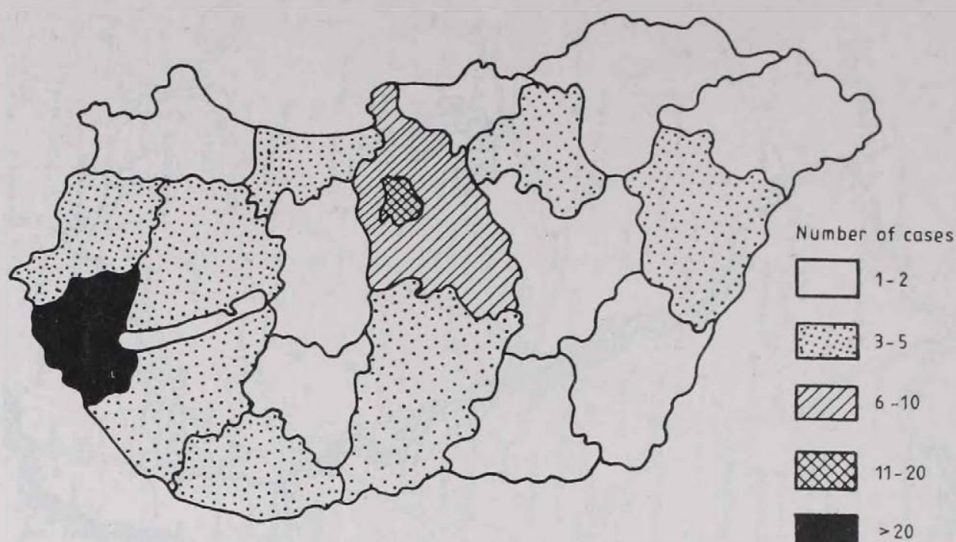


Fig. 8. Haemorrhagic fever with renal syndrome cases by county in Hungary, 1987–1994

Parasitic zoonoses

There is an important progress on the field of laboratory diagnostics of parasitic diseases (Table VII). About 500,000 human protozoological examinations are performed each year in the laboratories of the Public Health Service. Great part of them are directed to detect enteric parasites. On the basis of the results it can be stated that incidence rate of *G. lamblia* infections is 1.8% and that of *E. histolytica* infections is 0.4% [33]. It is thought that neither these protozoal infections nor shigellosis are real zoonotic diseases in our country.

Cryptosporidiosis is a newly recognized illness. The first case was diagnosed in 1986 [34]. Domestic and other animals may be the reservoirs. In a normal person the diarrhoea is self limiting. In an immunocompromised patient the organism can cause lethal disease because an effective therapy is not known.

Babesiosis was diagnosed among animals but human infection has not yet been verified in our country [12, 34]. In case of dogs ticks (*D. reticulatus*) were the transmitters [35].

Toxoplasmosis is a common zoonosis. More thousand serological examinations were done in 1994. The serum samples originated from suspected patients and from healthy pregnant women. Acute infection could be observed in 1.7–3.9% of pregnant women and chronic infection was demonstrated in 30–50% of them depending from their living district. The same positivity rates were observed among suspected patients, too [33]. These figures mean – knowing that the number of deliveries is over 100,000 in each year – that the number of notified toxoplasmosis cases is underreported. The aim of the regular survey of pregnant women is to prevent congenital toxoplasmosis cases. Acute cases can be treated with pyrimethamine and sulphonamides, spiramycine or clindamycine. Cat, raw pork and raw beef may be the source of the infection.

Taeniasis is a rare disease. Pigs are the intermediate hosts for *T. solium* and oxen are for *T. saginata*. The final host is that person who eats raw pork or beef containing cysts of the worm. At slaughterhouses there is a veterinary control. Pork and beef which are heavily infected by cysts are not suitable for human consumption. In case of a light infection the meat can be consumed only after heat treatment done at the slaughterhouse. To prevent the infection of intermediate hosts people must keep hygienic prescriptions.

In Hungary only *E. hydatidosus* infection can rarely be diagnosed. On the basis of the results of some 4079 necropsies less than 0.5% of the population may be infected [12]. Dogs are the main hosts. Human beings can be infected by *Echinococcus* eggs when they are in close connection with an excreter dog. Intermediate hosts are pigs kept and slaughtered in small farms, where there is no satisfactory veterinary control and when a pig harbours cysts the slaughter cut them out and usually throws them to dogs to eat.

Table VII

Parasitic zoonoses cases in Hungary, 1972-1994

Disease (Order of notification was issued)	Number of cases in years			Total number of cases
	1972-81	1982-91	1992-94	
Cryptosporidium	The first case was in 1986			
pfaeces (-)a	unk.	339b	98	437
Echinococcosis (1958)	71c	145c	21	237c
Schistosomiasis (1966)	71d	89d	3d	163d
Taeniasis (1958)	583c	382c	18c	983c
Toxocariasis sp. (-)	The first case was in 1979			
	unk.	3947	2295	6242
Toxoplasmosis sp. (1966)	514	2004	458	2976
Trichinellosis (1930)	150	174	2	326

Occasional diseases: A few human liver flukes caused by *F. hepatica* were diagnosed. Imported cutan leishmaniasis was diagnosed

pfaeces = positive faeces

a = by Kinyoun acid-fast stain

sp. = serologically positive

b = data of some years are missing

unk. = unknown

c = imported cases are included

d = imported cases

Trichinellosis is also a rare disease because there is an obligatory veterinary control at slaughterhouses and keeping conditions of pigs are generally suitable. The cause of all diagnosed cases was the illegal use of meat of wild pig to prepare smoked sausages.

Trichinellosis has been a reportable disease since 1947 in the U.S. The number of cases reported annually has declined from an average of 400 cases per year in the late 1940s to 32 cases in 1994. The proportion of cases associated with eating contaminated commercial pork has been declined since 1975, most likely because of laws prohibiting feeding hogs with offal. The increased use of home freezers and the practice of thoroughly cooking pork are also important. The proportion of cases associated with eating wild game has increased and cases have resulted from consumption of bear, wild boar, walrus and cougar. Although most species of trichinellae are killed by freezing, freeze-resistant strains can also be found. To ensure that trichinellae are destroyed, meat should be thoroughly cooked. A temperature of 77 °C exceeds the thermal death of the trichinellae [36].

The number of pet animals has also increased in our country similarly to the most industrialized countries. Pet keepers and breeders can more easily be infected by zoonotic pathogens among them by *Toxocara* spp. These parasites are common in dogs and cats, therefore their environment are frequently contaminated with *Toxocara* eggs. Children can freely take the eggs in a contaminated environment. It was observed in our country during a survey that seropositivity was more frequent in children who lived in low socioeconomic and rural conditions [34]. It seems that a symptom-free toxocariasis may lead to neurological deficit, epilepsy or behaviour disorders in children, therefore this infection deserves attention [18]. Toxocariasis can be prevented by careful personal hygiene, elimination of intestinal parasites from pets, protection of play grounds of children from contamination and education of pet owners, breeders and sellers.

Table VIII

Zoonotic dermatophytosis cases in Hungary, 1972–1994

Pathogens (Order of notification)	Number of cases in years			Total number
	1972–81	1982–91	1992–94	
<i>M. canis</i> (–)	395	3103	1476	4974
<i>M. vanbreuseghemii</i> (–)	0	3	2	5
<i>T. equinum</i> (–)	1	0	5	6
<i>T. mentagrophytes</i> (–)	2876	1749	492	5117
<i>T. verrucosum</i> (–)	82	168	36	286

The figures mean the number of strains isolated from samples

Zoonotic dermatophytosis

It is very difficult to judge the real importance of zoonotic dermatophytosis because there is no obligatory data-collection for these diseases. The number of laboratories which voluntarily report the data alters year to year. Besides, only those cases are reported which are diagnosed with cultivation but this method is not compulsory. It is also a problem that fungi can hardly be classified strictly into anthropophil, zoophil and geophil groups (Table VIII). On the basis of the opinion of our experts the number of *M. canis* infections is substantially greater than shown in Table VIII. Increasing number of pets has enhanced importance of this pathogen. *M. vanbreuseghemii* strain is originally geophil but in these cases which are presented here the infection originated from a cat. *T. equinum* is a zoophil species but in contrast with this in 1993 an epidemic started among wrestlers and the pathogen spread from man to man. *T. mentagrophytes* can infect both human beings and animals. As the source of the infection cannot be verified in all instances, the figures may represent not only zoonotic strains [37].

Occupational zoonotic illnesses

Notification of occupational diseases is obligatory. On the basis of the Order of Minister of Health anthrax, brucellosis, echinococcosis, erysipelas, leptospirosis, Lyme borreliosis, ornithosis, Q-fever, tick-borne encephalitis, toxoplasmosis, trichophytiasis, tularemia, yersiniosis and other zoonoses must be reported [11]. Those persons who got occupational zoonoses may be compensated. It is a problem that doctors insufficiently report occupational diseases. That can be concluded when the number of reported occupational cases, that of epidemiologically surveyed occupational cases and the number of notified infectious diseases are compared [38, 39] (Table IX). Those infections which are presented in the last rows of the column of Table IX are only notifiable occupational zoonoses but they are not designated as notifiable infectious diseases.

The incidence rate of occupational zoonoses is different in single counties (Fig. 9). The highest rate was observed in Békés county which was followed by Baranya, Somogy, Zala and Vas counties. The employees of big meat industrial factories situated in these counties got most frequently occupational infections. Their disease was mainly erysipelas [39]. It is interesting to mention that 18 cases of brucellosis among employees at a local porc processing plant were diagnosed from November 1991 through September 1992 in the U.S. During the survey of the plant of the 156 workers in the kill division 30 (19%) met the case definition for brucellosis, 16 (53%) with previously unrecognized cases. The findings indicate that occupational transmission of brucellosis remains a public health hazard, particularly among persons exposed to swine [13].

Table IX

Zoonoses cases by way of report in Hungary 1990-1994

Disease	Notified infectious diseases	Occupational cases by	
		epidemiological survey	report of MD
Anthrax	2	2	2
Brucellosis	8	4	2
Leptospirosis	191	83	53
Ornithosis	7	1	0
Q-fever	26	5	3
Taeniasis	46	3	0
Tick-borne encephalitis	1330	147	95
Toxoplasmosis	813	34	0
Trichinellosis	9	3	0
Tularemia	292	43	18
Total	2769	323	172
Erysipeloid	0	0	
Lyme borreliosis	0	0	333
Milkers node	0	0	
Pyogenic infections	0	0	105
Trichophytiasis	0	0	104
Other fungal infections	0	0	38

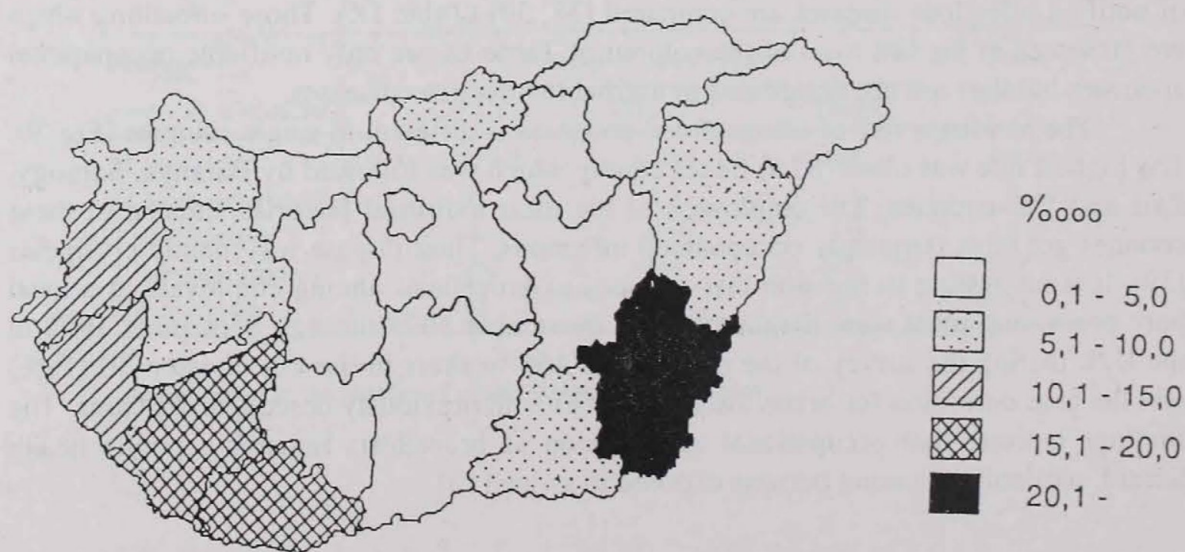


Fig. 9. Rate of occupational zoonoses cases by county in Hungary, 1990-1994

Table X

Occupational zoonoses cases by industries in Hungary 1990–1994

Disease	Agriculture and forestry	Food and tobacco	Water supply	Other	Total number	In %
Anthrax	2	0	0	0	2	1.2
Brucellosis	2	0	0	0	2	1.2
Leptospirosis	23	23	2	5	53	30.6
Q-fever	3	0	0	0	0	1.7
Tick-borne encephalitis	85	1	2	7	95	54.9
Tularemia	16	1	0	1	18	10.4
Together	131	25	4	13	173	100.0

The most occupational zoonoses cases in Hungary were diagnosed among the workers of agriculture and forestry (Table X). Employees of food and tobacco industries stay on the 2nd place. Flaviviruses which are responsible for tick-borne encephalitis, leptospirae and *F. tularensis* are the most frequent microorganisms causing notifiable occupational infections besides pyogenic streptococci. They are followed by *C. burnetii*, *B. anthracis* and *brucellae* [39].

It seems from the data that relative importance of occupational zoonoses has increased during last years.

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HUMAN PAPILLOMA VIRUSES IN NON-MELANOMA SKIN CANCERS

(A SHORT REVIEW)

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The knowledge in the realms of pathology, epidemiology and molecular biology of human papillomaviruses (HPV) has defined them as etiological agents in benign tumors of the anogenital tract and major carcinogens in cancer of cervix uteri.

Non-melanoma skin cancer (NMSC) is the most common human cancer amongst lightly pigmented individuals. The mortality is low, the morbidity is significant in susceptible individuals often developing multiple primary tumors.

Several groups now report a high prevalence of HPV DNA in human NMCS of immunosuppressed patients. This provides impetus for researching the role (causal or passenger) of cutaneous HPVs in the genesis of skin cancer.

Epidermodysplasia Verruciformis

Epidermodysplasia Verruciformis (EV) is an extremely rare skin disease, which is a model of HPV-induced cutaneous malignancy. EV is characterized by a genetic predisposition to infection with specific oncogenic HPV types, including HPV 5, 8, 14, 17, 20, 47.

Predominantly types 5 and 8 are found in over 90% of these skin cancers [1]. EV patients have a specific inability to control HPV infection at the level of the keratinocyte. EV patients develop widespread warts on all body sites, including hands, face, trunk and legs [2]. The so-called EV skin cancers as squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) may appear over 2 decades later (average time 24.5 years) after the onset of wart infection [3].

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Tumors occur almost exclusively on sun-exposed body sites and are much less common in black EV patients, suggesting that UV light is a critical co-factor [4].

The strong association of HPV 5 and 8 DNA with EV-associated cutaneous SCC equals with that seen between HPV 16/18 and cervical cancer.

In contrast to cervical cancer, where viral DNA frequently integrates into the host genome, HPV 5 and 8 in EV-associated cutaneous SCC is usually present as episomal DNA in high copy numbers (100–300 per diploid cell). The transforming potential of EV HPV types appears lower than that of the high risk mucosal HPV type [5, 6].

Current evidences suggest different cellular targets for EV HPV oncoproteins than those (cellular suppressor proteins, retinoblastoma susceptibility gene product) identified in cervical cancer. The mechanisms involved in EV-associated skin cancer remain to be determined.

However the HPV 5 and type 8 contain sequences (within the viral regulatory region) with homology to an UV-responsive element, which suggests that UV-induced cellular proteins may directly regulate EV HPV transcription [7].

HPV detection in skin cancers

A large number of mucosal, but relatively few cutaneous HPV types have been identified. HPV types are classified according to similarities in special genomic sequences. These are the two oncogenes – early 6 (E6) and early 7 (E7) and a gene (L1) with a coding capacity of the major viral capsid protein [8]. The L1 region is the most highly conserved region of the papillomavirus genome. A new HPV type can be defined when less than 90% homology of the L1 DNA sequences are determined. The HPV detection techniques used in NMSC are: Southern blot, in situ hybridization and different types of DNA polymerase chain reactions (PCRs with consensus, type specific, degenerate primers).

Data of prevalence of HPV in cutaneous lesions has been summarized comprehensively recently [9]. These studies using probes for common genital and common cutaneous HPV types found HPV DNA in only 2 of 76 (3%) SCCs and 1 of 53 (2%) BCCs [10]. The general result of these works is the demonstration of a low prevalence of HPV 16 and a virtual absence of HPV 5 and 8 in NMSC. In contrast HPV 16 is present in over 60% of cervical cancers and HPV 5 and 8 in over 90% of EV [11, 1]. An exception to this general rule are two rare types of skin cancer where HPV 16 may play a role in the development of malignancy. These are invasive and in situ squamous tumors occurring adjacent to the nail and on the palms or soles (periungual and palmoplantar SCC and Bowen's disease). HPV 16 was found in 13 of 21 (60%) cases of periungual SCCs and in 14 of 21 (67%) cases of periungual and palmoplantar Bowen's disease [12, 13]. Genital transmission (autoinoculation?) of virus via the fingers may be implicated, since – in some cases – HPV 16 in both skin tumor tissue and cervical tissue can be demonstrated [14].

There are reports of unusual HPV types in malignant or premalignant cutaneous tumors from the general population. The most notable of these is HPV 41 [15].

HPV detection in skin cancers from immunosuppressed transplant recipients

An association between warts with HPV etiology and skin cancer in renal transplant recipients (RTRs) was first noted in Australia [16]. Very often RTRs have numerous benign and malignant skin tumors distributed all over the body (papillomas, actinic keratoses, basal and squamous cell carcinomas and Bowen's disease). In a study 17 formalin-fixed, paraffin embedded biopsies (benign and malignant skin tumors) of RTR were analysed for HPVs. Viral DNA was detected in 65% of the biopsies. Sequencing of cloned amplification products 9 different HPV types were identified, but four sequences differed from all HPVs known so far [17]. RTR patients have a well-documented increased risk of cutaneous SCC and a marked reversal in the BCC to SCC [16, 17]. The all-age cumulative incidence of skin cancer in transplant recipients is 27–44% after 10–25 years of immunosuppression [18].

Table I

Case series examining HPV DNA in cutaneous SCC from transplant recipients

Study area	Number of SCC	HPV DNA (%)	HPV types			Other
			Common cutaneous	Common EV-related HPV (HPV 5/8)	Common other mucosal	
France	46	25/46 (54%)	20/46	2/46	15/46	–
	26	21/26 (81%)	0/26	6/26	20/26	–
England	10	6/10 (60%)	2/10	2/10	0/10	Unknow HPV types in 2/10
England+ Germany	20	13/20 (65%)	0/	0/20	1/20	HPV 41, 54, 61, 69 and 7 novel HPV types
Scotland	25	16/25 (64%)	1/25	15/25	NT	–
	30	10/30 (33%)	3/30	0/30	2/30	Unknown HPV types in 6/30
Netherlands	24	5/24 (21%)	0/24	1/24	0/24	Unknown HPV types in 2/24 HPV 14 related types 2/24
	61	49/61 (80%)	0/61	0/61	0/61	HPV 15, 19, 20, 21, 23, 24, 25, 38 and 6 novel types

SCC = Squamous cell carcinoma

NT = Not tested

Virus warts and cutaneous SCC on sun-exposed sites and ultraviolet light appears to be an important factor in the development of both warts and cancers. Benign virus warts in RTRs are associated with a much more diverse range of HPVs than found in benign warts from the normal population [19]. Also many skin lesions of RTRs contain multiple infections with more than one HPV type.

Recent studies demonstrate a high prevalence (65–80%) of HPV DNA in dysplastic and malignant skin tumors from RTRs. HPV types identified include: EV HPV types 9, 14, 15, 17, 19, 20, 21, 23, 24, 25, 36 and 38; high risk mucosal HPV types 16, 51, 54, 56, 61 and 69; and cutaneous HPV types 27, 41, 49 and 60 [20, 21] (Table I).

The prevalence of these broad spectrum of HPV types in skin lesions is not yet known. In both anogenital cancer and EV-associated skin cancer there is a specific association of certain HPV types with transformation: HPV16, 18 in anogenital- and HPV 5, 8 in EV cancers. It remains of interest to determine whether there are cutaneous HPV types specially associated with malignant lesions.

Future research on the role of HPV in NMSC might be reflected on searching for specific cellular targets and identifying viral oncoproteins. In functional studies the combined effects of viral oncoproteins should be examined with ultraviolet light.

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EXTRACELLULAR CELLULASE SYSTEM OF A THERMOTOLERANT STREPTOMYCETE: *STREPTOMYCES ALBADUNCUS*

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During screening for cellulolytic streptomycetes from different natural substrates, *Streptomyces albaduncus* thermotolerant streptomycete was selected for detailed studies because of its comparatively better yield and ability to grow at higher temperatures (up to 47 °C). The production of cellulases of this strain was studied under different growth conditions. It produced optimum levels of all the three constituents of cellulase complex at pH 7.0, and 37 °C in the presence of 3% substrate. When grown as submerged culture under these conditions, the strain produced considerable amounts of filter paper degrading activity (FPase), carboxymethyl cellulose hydrolyzing activity (CMCase) and β -glucosidase activity in the extracellular supernatant. It exhibited good overall cellulolytic activity as measured using cotton as substrate. The maximum yields of FPase and CMCase were obtained within 96–120 h and β -glucosidase after 48 h. The optimum incubation temperature for enzyme activities was 50 °C and optimum pH in the range of 6.0–6.5. After analyzing the cellulase complex of *S. albaduncus*, we are now capable to improve it genetically for superior yields.

The complete hydrolysis of cellulose is thought to be the result of synergistic action of the constituents of cellulase enzyme complex viz. an endo-1,4- β -glucanase (E.C. 3.2.1.4) which cleaves the polymer in a random fashion to produce free chain ends, an exo-1,4- β -glucanase (E.C. 3.2.1.91) which cleaves cellobiose units from the non-reducing chain ends and a glucosidase (E.C. 3.2.1.21) which hydrolyzes cellobiose to glucose. Although streptomycetes have been shown to be highly efficient in degrading lignocellulose [1–4], comparatively few cellulase systems from *Streptomyces* species have been studied in any detail [5–8]. In a search for cellulose degrading microorganisms, 114 cellulolytic streptomycetes were isolated from various natural habitats. Because of comparatively higher cellulase activity and thermostability of its glucanases as compared to other organisms especially hyperproducer *Trichoderma reesei*, the cellulase system of

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Streptomyces albaduncus, a mushroom compost isolate was studied in detail. In this paper optimum conditions for the production and activities of cellulases of this strain are reported.

Materials and methods

Streptomyces strains and culture conditions. *Streptomyces* strains were obtained from different sources on glycerol-arginine medium [9], purified and identified as *Streptomyces* species by using standard procedures described in the International *Streptomyces* Project (ISP) published by Shirling and Gottlieb [10] and Bergey's Manual of Systematic Bacteriology [11]. The strain of *S. albaduncus* used for further studies was procured from mushroom compost and deposited in Microbial Type Culture Collection (MTCC No. 1764) of Institute of Microbial Technology, Chandigarh (India). The stock cultures were maintained in soil at 4 °C and whenever required fresh culturing was done on glycerol-arginine agar slants.

The basal medium used in the submerged culture studies had the following composition: KH_2PO_4 , 1.5 g; K_2HPO_4 , 2.0 g; $(\text{NH}_4)_2\text{SO}_4$, 1.4 g; yeast extract, 2.0 g; proteose peptone, 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.03 g; Tween 80, 2 ml; trace metals solution [12], 1 ml. The volume was made up to 1000 ml with water and pH adjusted to 7.0 with NaOH. To induce cellulase production, 10 g of microcrystalline cellulose (MCC) was added. Growth and culture studies were carried out on a rotary shaker (G 24 New Brunswick Scientific Co., New Jersey, USA) at 250 rpm.

Fur inoculum build up, 100 ml of basal medium supplemented with 1% glucose was inoculated with spore suspension obtained by adding 5 ml of physiological saline, containing 1% Tween 80, to a 7 day old slant culture and incubated at 37 °C for 24 h. This culture was centrifuged at 5,000 rpm for 10 min. The pellet was resuspended in 50 ml of the medium and used as the inoculum (at 2% concentration) for these studies.

Optimization of cultural conditions for cellulase production. To determine the optimum temperature for cellulase production, shake cultures at pH 7.0 were incubated in the temperature range of 27–47 °C. The samples were analyzed for enzyme production at 24 h intervals for 144 h.

The optimum pH values for extracellular cellulase production were determined by incubating shake cultures in MCC containing medium in the pH range of 4.5–10.0. In a separate experiment the substrate (MCC) concentration varied from 0.5–4.0%. In both cases flasks were incubated at 37 °C and were analyzed after 48 h for β -glucosidase and after 96 h for FPase and CMCase.

Enzyme preparation. Shake cultures were centrifuged at 10,000 g for 30 min at 4 °C and the supernatant obtained was used to estimate extracellular enzymes.

Cellulase assays. Cellulase activities were assayed by measuring the release of reducing sugars (RS) by the dinitrosalicylic acid (DNS) method of Miller et al. [13] with glucose as standard.

Filter paper activity (FPase). The enzyme activity was measured by adding 0.9 ml of 0.05M citrate buffer (pH 6.0) and 0.1 ml of appropriately diluted enzyme to 50 mg of Whatman No. 1 filter paper (1×6 cm strip). The reaction mixture was incubated at 50 °C for 1 h and the amount of RS produced was determined.

β -1,4-Glucanoglucanohydrolase (E.C. 3.2.1.4, CMCase). The enzyme was estimated by incubating the tubes containing 0.1 ml of suitably diluted enzyme extract, 0.4 ml of 0.05M citrate buffer (pH 6.0) and 0.5 ml of 1% carboxymethyl cellulose (CMC, d.s. 0.7, d.p. 400, low viscosity) solution in the same buffer. After incubation for 20 min at 50 °C the reducing sugar content was determined.

Cotton activity. The cotton degrading activity was measured by incubating 0.9 ml of 0.05M citrate buffer (pH 6.0) with suitably diluted culture filtrate and 50 mg of dewaxed surgical absorbent cotton for 12–60 h at 50 °C and the amount of RS released was measured.

The reducing sugars already present in the enzyme samples were also considered before calculating the actual amount of glucose released.

β-Glucosidase activity (E.C. 3.2.1.21). The activity was measured by incubating a reaction mixture consisting of 0.9 ml of 0.01M p-nitrophenyl-β-D-glucopyranoside (pNPG) in 0.05M sodium potassium phosphate buffer (pH 6.5) and 0.1 ml of enzyme preparation at 45 °C for 30 min. The reaction was stopped by adding 4 ml of 0.2M NaOH/glycine buffer (pH 10.6). The yellow coloured p-nitrophenol was determined spectrophotometrically at 420 nm from a standard curve using p-nitrophenol.

All enzyme units were expressed in μ moles product released per min. Cotton activity was expressed in mg of reducing sugar (RS), as glucose, in 1.0 ml supernatant produced under the assay conditions.

Results

Characteristics of the streptomycete. *S. albaduncus* strain MTCC 1764 produces incomplete or imperfect spirals. The spores have a spiny surface as determined by electron microscopy. It produces grey spores in oat meal agar, glycerol-asparagine medium and glycerol-arginine agar. It did not sporulate in peptone-yeast extract iron agar but produced a leathery colony. Diffusible pigment was not produced in the following media: oat meal agar, peptone-yeast extract iron agar and tyrosine agar. The strain grows optimally at 40 °C with temperature range of 26–47 °C. The significant growth observed at 47 °C showed *S. albaduncus* to be thermotolerant.

The analysis of whole cell hydrolysate by thin layer chromatography (TLC) revealed the presence of L,L-diaminopimelic acid and sugars (glucose and xylose).

The carbohydrates used for the growth were: D-glucose, L-arabinose, D-xylose, i-inositol, D-mannitol, D-fructose, rhamnose, salicine and ribose. It did not utilize raffinose and only a trace of growth was observed with sucrose. On the basis of these results, the strain has been identified as *S. albaduncus*.

Optimal cultural conditions for cellulase production. Maximum activity levels of all the three components were obtained at 37 °C (Fig. 1). FPase and CMCase activities increased rapidly during the first 72 h, after which the increase was gradual till the termination of the experiment. β-Glucosidase production displayed different patterns at different temperatures. It showed two peaks each at 32, 37 and 42 °C. The first peak at all these temperatures was registered after 48 h, while the second one after 96 h at 37 °C and after 120 h at 32 °C and 42 °C. At 47 °C, the highest levels were recorded after 24 h. Lower cellulase production occurred at 32, 42 and 47 °C as compared to 37 °C while at 27 °C very low enzyme levels were produced.

The optimum pH for the synthesis of all the three cellulase enzyme activities was found to be 7.0. Both FPase and CMCase showed a good activity at pH 5.0–8.5. In contrast, β-glucosidase was produced in a narrow range i.e. at pH 6.0–7.0.

Maximum cellulase production was observed with 3% MCC, higher concentrations being slightly inhibitory to FPase and CMCase. In contrast, the increase in β-glucosidase production was linear up to 3% and became constant on further increase of substrate concentration.

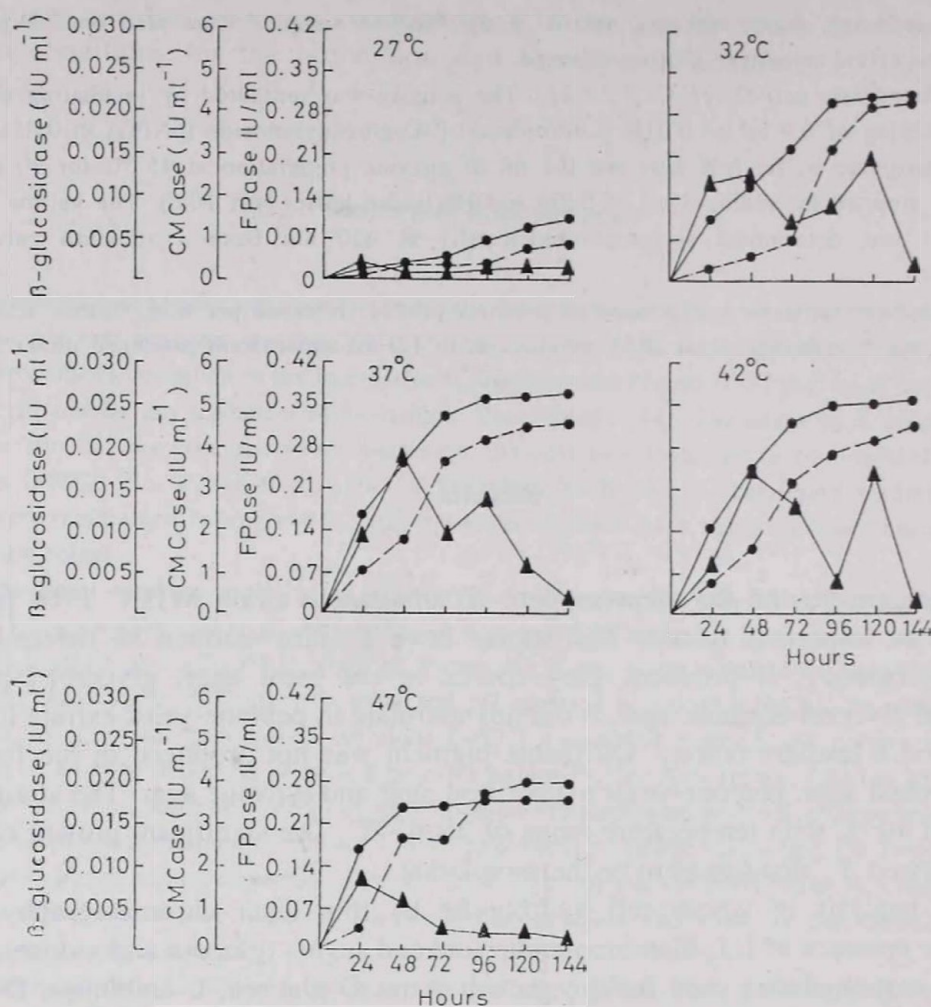


Fig. 1. Effect of incubation temperature on the extracellular FPase (● --- ●), CMCase (● — ●) and β -glucosidase (▲ — ▲) production in *S. albaduncus* when grown in shake flasks containing basal medium with 1% MCC as substrate

The pattern of pH change and cellulase production by *S. albaduncus* when grown under optimum conditions (pH 7.0, 3% MCC at 37 °C) is shown in Fig. 2. The pH of the culture filtrate dropped gradually to 6.2 from original pH 7.0 during 48 h and then stabilized till 144 h of cultivation. Considerable levels of extracellular FPase and CMCase were obtained after 24 h, followed by a regular increase yielding 0.472 IU/ml and 6.375 IU/ml, respectively, at 144 h. The maximum production of glucosidase (0.056 IU/ml) was detected after 48 h followed by a regular decline.

Proteolytic inactivation was apparently not a significant factor in the reduction of β -glucosidase activity when cultures containing higher levels of proteases were mixed with β -glucosidase from 48 h cultures at pH 6.2 and incubated at 37 °C for 10–20 min, inactivation of this enzyme was equal to that of control experiments. In contrast, FPase and CMCase were slightly activated (data not shown).

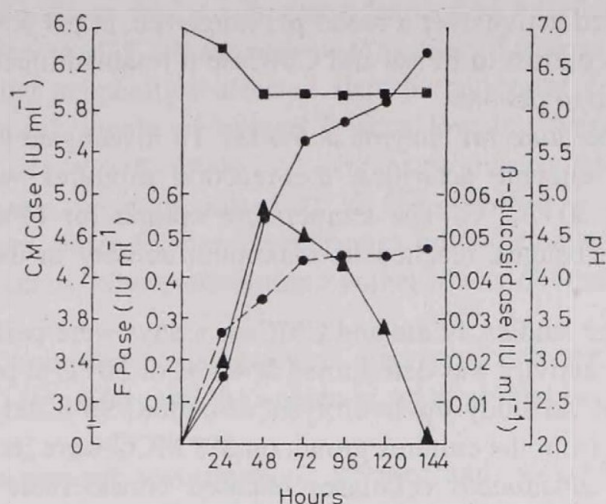


Fig. 2. Production of extracellular cellulases by *S. albaduncus* when grown in shake flasks containing basal medium with 3% MCC at 37 °C. FPase (● — ●), CMCase (● — ●), β-glucosidase (▲ — ▲) and change in pH (■ — ■)

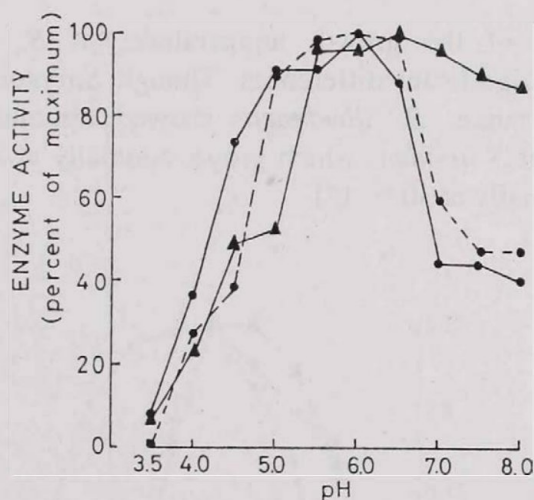


Fig. 3. Effect of pH on the extracellular FPase (● — ●), CMCase (● — ●) and β-glucosidase (▲ — ▲) activity of *S. albaduncus*

Optimum pH for enzyme activities. For the determination of the optimum pH for extracellular cellulase activities the enzyme assays were performed in the pH range of 3.5–8.0 (using 0.05M citrate buffer for pH 3.5–6.5, and 0.05M phosphate buffer for pH 6.5–8.0). The optimum pH value for FPase and CMCase was pH 6.0 (Fig. 3). Their activities decreased rapidly at pH 7.0 and 4.0 with only trace amounts at pH 3.5.

β -Glucosidase remained active over a broad pH range (i.e. at pH 5.5–7.0) with maximum activity at pH 6.5. In contrast to FPase and CMCase it retained much of its activity in the alkaline range, even up to pH 8.0.

Optimum temperature for enzyme activities. To investigate the optimal incubation temperatures for the enzyme activities, the reaction mixtures were incubated in the temperature range of 30–70 °C. The temperature optima for FPase and CMCase was 50 °C, whereas β -glucosidase reached its maximum activity in the range of 45–50 °C (Fig. 4).

Thus, for further studies, FPase and CMCase assays were performed at 50 °C at pH 6.0 and β -glucosidase activity was determined at 45 °C or 50 °C at pH 6.5.

Cotton activity. To study the hydrolysis of cotton, 96 h old extracellular enzyme preparations obtained from the cultures grown on 3% MCC were incubated with dewaxed absorbent cotton. *S. albaduncus* cellulases released considerable amount of reducing sugars after 12 h (Fig. 5). Maximum saccharification of cotton was observed after 24 h showing the release of 1.38 mg RS/ml. With further incubation, which was followed for 60 h, the amount of RS released started to decrease slightly.

Discussion

The comparison of the growth temperatures of *S. albaduncus* with other streptomycetes showed significant differences. Though *Streptomyces lividans* grows in the same temperature range, *S. albaduncus* showed optimum growth at a higher temperature (40 °C) than *S. lividans* which grows optimally at 27 °C [7]. *Streptomyces flavogriseus* grows optimally at 30 °C [5].

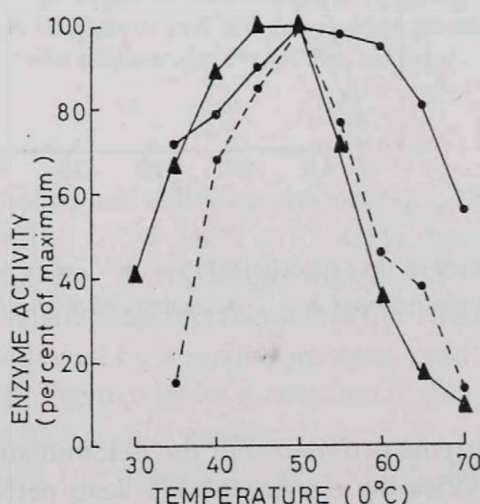


Fig. 4. Effect of temperature on the extracellular FPase (● — ●), CMCase (● — ●) and β -glucosidase (▲ — ▲) of *S. albaduncus*

The cultural conditions had a significant impact on cellulase formation. All the three cellulase constituents of *S. albaduncus* were produced maximally at 37 °C and pH 7.0. It was apparent that temperature affected also the pattern of cellulase formation. At 47 °C although the peak levels of enzymes were low but were obtained earlier as compared to low temperatures. Similarly, *S. lividans* produced maximum endoglucanase after 48 h when cultures were incubated at 37 °C, whereas at 32 °C after 72 h of incubation [7]. However, other *Streptomyces* species followed patterns that were different from that of *S. albaduncus*, where maximum synthesis was obtained at 30 °C [5, 8, 14, 15].

Most of the actinomycetes and bacteria are reported to produce cellulases at or around pH 7.0 [5, 14, 16]. However, the values in fungi ranged between pH 4.0–5.0 [17, 18].

Similar to the present investigation, Bernier and Stutzenberger [19] reported maximum β -glucosidase accumulation at higher concentrations (i.e. up to 4%) of substrate in *Thermomonospora curvata*. In contrast to these findings, there are reports which demonstrated higher concentrations to be inhibitory to the enzyme production [18, 20]. This decreased enzyme activity at elevated substrate concentrations can be attributed to the adsorption of enzymes on unutilized substrate [21] or increased levels of reducing sugars accumulated through saccharification of the substrate which may have repressed the cellulase synthesis [22].

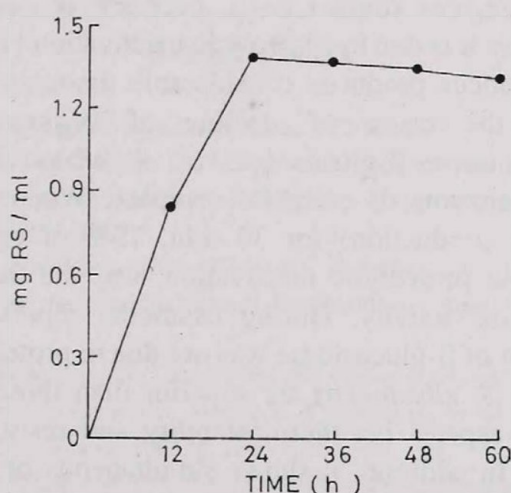


Fig. 5. Release of reducing sugars (RS) from 50 mg of cotton incubated with buffered (pH 6.0) 96 h old extracellular enzymes of *S. albaduncus*

During cellulase production in various microorganisms variable pH patterns have been observed. pH of the carboxymethyl cellulose containing culture of *Aspergillus nidulans* increased rapidly from pH 5.0 to 8.0, with maximum growth and cellulase production at pH 8 [18]. Cultures of *Thermoactinomyces* strain used by Hagerdal et al. [23], *S. flavogriseus* by Ishaque and Kluepfel [5], streptomycetes by Van Zyl [14] and

streptomycete used here showed a decrease in pH, producing bulk of cellulases between pH 6.8 and 6.1. The pH change is probably due to the uptake of NH_4^+ ions, since actinomycetes are highly oxidative [24] and do not release acids to the medium.

The overall production of cellulase by *S. albaduncus* as determined by filter paper test reached 0.432 IU/ml after 72 h. This can be favourably compared with values obtained by the use of original *T. reesei* strain QM 6a [25] and *S. flavogriseus* [5]. However, in the streptomycetes cultures these extracellular enzyme levels were obtained in 3 days, whereas in *T. reesei* maximum yield occurred after 7 days [26]. *S. albaduncus* produced 0.268 IU/ml of the same enzyme after 24 h which is comparable to the values obtained from *Thermoactinomyces* in the same period [23]. However, in *S. albaduncus* the production increased continuously by further cultivation, whereas in *S. flavogriseus* it becomes stationary and in *Thermoactinomyces* it starts decreasing.

On the other hand, overall production of CMCase after 72 h was 5.47 IU/ml which is about 50% of the activity of *Thermoactinomyces* [23], 18–23% of the activities of *S. flavogriseus* [5] and the cellulases produced by three mesophilic actinomycetes studied by Van Zyl [14] and five times the yield of cellulases produced by *S. lividans* [7]. *S. albaduncus* produced maximum β -glucosidase (0.056 IU/ml) after 48 h which is lower than the enzyme activities produced by *S. flavogriseus* (0.072 IU/ml). However, the latter produced these levels after 72 h, so the lower yields are compensated by the rapid production.

A rapid decline in β -glucosidase activity of culture fluid was observed after 48 h. This type of decline has been observed in other actinomycetes. In *Streptomyces flavogriseus* and *Streptomyces* strain CB-12, decrease is due to fall in pH whereas *Thermomonospora curvata* it is due to proteolytic inactivation [19, 27].

Though *S. albaduncus* produces considerable amounts of proteases, proteolytic inactivation was not the cause of decline of β -glucosidase activity. During characterization of cellulases, β -glucosidase of *S. albaduncus* was found to be a thermostability-limiting enzyme of cellulase complex. When held at 37 °C (optimum temperature for enzyme production) for 30 min, 75% of enzyme was lost [28]. So thermolability, rather than proteolytic inactivation, was the major factor governing the extracellular β -glucosidase activity. During catabolite repression studies it was also observed that inactivation of β -glucosidase was not due to proteolysis [29].

The cellulases of *S. albaduncus* are superior than those of the above-mentioned streptomycetes in other aspects i.e. thermostability and resistance of β -glucosidase to glucose inhibition [28]. In addition, it shows simultaneous production of commercially useful enzymes i.e. proteases [29], amylases and xylanases (unpublished data) in significant amounts. Based on these observations *S. albaduncus* can be regarded as a cellulase producer of considerable economic potential and can be further improved genetically for better production.

The overall cellulolytic activity is measured by the degradation of filter paper and cotton which are most resistant to biodegradation. The cellulase of *S. albaduncus* not only caused a significant hydrolysis of filter paper but also caused the hydrolysis of cotton more rapidly and for a longer duration as compared to *S. flavogriseus* [5]. Thus, the data show that the cellulase of *S. albaduncus* used in these studies contained the complete enzyme system.

The optimum pH for the assay of FPase and CMCase was 6.0 whereas β -glucosidase showed maximum activity at pH 6.5. These values are in agreement with the general range for many actinomycetes but somewhat higher than those reported for fungi. The optimum pH in fungi lies around 4.5 and 5.5 [30] whereas the optimum pH for streptomycetes cellulases proved to be 5.0–7.0 [5, 14, 31]. However, several cellulases with alkaline optimum pH have also been reported in alkalophilic *Bacillus* strains [32] and *Streptomyces* strain KSM-2 [8].

FPase and CMCase of *S. albaduncus* showed maximum activities at 50 °C. This value is very close to the temperatures observed for glucanases of some mesophilic fungi [30] and actinomycetes [5, 7, 14, 33]. However, these values are 5–10 °C lower than those reported for *Thermomonospora fusca* [34] and *Microbispora bispora* [35], both of which are considered to be thermophilic actinomycetes.

In the present study β -glucosidase of *S. albaduncus* MTCC 1764 showed maximum activity in the temperature range of 45–50 °C. The β -glucosidase of *S. flavogriseus* and *S. lividans* HP-3 were shown to be maximally active at 40 °C [27, 31] whereas the optimum temperature for *Streptomyces* strain W19-1 was manifested to be 60 °C [36].

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STUDIES ON ANTIMICROBIAL EFFECT OF THE ANTIHISTAMINIC PHENOTHIAZINE TRIMEPRAZINE TARTRATE

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The antibacterial and bactericidal activities of the antihistamine trimeprazine were studied against 243 strains of bacteria which included both Gram positive and Gram negative types. The susceptibility of these bacterial strains to trimeprazine was assessed by determining their minimum inhibitory concentration (MIC) which was found to be between 10 and 100 µg/ml. Nineteen strains of *Staphylococcus* spp. were inhibited at 10–50 µg/ml of trimeprazine. Most of the strains belonging to *Bacillus* spp. and *Salmonella* spp. were inhibited by less than 100 µg/ml. Trimeprazine could also inhibit strains of *Shigella* spp. *Vibrio cholerae* and *V. parahaemolyticus* at 10–100 µg/ml. Strains of *Klebsiella*, *Proteus*, *Pseudomonas* and *Citrobacter* were moderately sensitive to trimeprazine.

In *in vivo* studies it was seen that when trimeprazine was used at a concentration of 0.75 and 0.4 µg/gm body weight of the mouse both levels offered significant protection to Swiss mice challenged with 50LD₅₀ of virulent strain of *S. typhimurium* 74. Statistical analysis of the data was found to be significant, $p < 0.001$ according to χ^2 test.

Conventionally, a drug is designated by its dominant or the first recognised function, however, after their usage some other effects could be discovered either accidentally or by systematic research. Thus powerful antimicrobial action of the psychotropic chlorpromazine [1–4] and promazine [5], of the antihistaminic diphenhydramine and bromodiphenhydramine [6], methdilazine [7, 8], promethazine [9] and fluphenazine [10], of the antihypertensive propranolol [11] and methyl-DOPA [12]

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and also of the local anaesthetic lignocaine and procaine [13] has been observed. The antimicrobial activity of these agents could be further potentiated by successful synergistic combinations with existing antibiotics and known antibacterial chemotherapeutics [14, 15]. It is evident from the above studies that phenothiazines represent a unique class of compounds, possessing remarkable antimicrobial potency along with antitumour and antiplasmid activities [16–18]. The present study deals with the in vitro and in vivo evaluation of the antibacterial activity of an other phenothiazine, namely trimeprazine, an antihistamine.

Materials and methods

Bacteria. Two hundred and forty-three strains of bacteria belonging to 12 different genera were used. The strains originated from various sources as described earlier [19]. These were all maintained as stab slant cultures at 4 °C and also in freeze-dried state. The drug trimeprazine tartrate was obtained in pure dry powder form from Sarabhai Chemicals, Bombay.

Determination of Minimum Inhibitory Concentration (MIC) of trimeprazine. Gram negative bacteria were grown in peptone water (P W, 1% bacteriological peptone, (Oxoid brand) plus 0.5% Analar NaCl) and the Gram positive ones in nutrient broth (N B, Oxoid) for 18 h. An aqueous stock solution of the drug trimeprazine was made at a concentration of 2 mg/ml and was sterilized by filtration through a G-5 glass filter, and stored at 4 °C. For spot inoculation test the drug was mixed at concentrations of 0 (control), 5, 10, 25, 50, 100 and 200 µg/ml to molten nutrient agar (Oxoid) at 50 °C. The pH of the media was maintained at 7.2–7.4. The MIC of trimeprazine was detected by spotting one 2 mm (internal diameter) loopful of an 18 h old broth culture containing approximately 10⁶ colony forming units (C.F.U.) on all the plates. All these plates were incubated at 37 °C and examined for growth up to 72 h.

Determination of bactericidal/bacteriostatic activity of trimeprazine tartrate (Sarabhai Chemicals, Bombay). Bactericidal/bacteriostatic activity was studied by the use of 18 h old cultures of sensitive bacteria. Two ml of the suspensions was added to 4 ml of a fresh nutrient broth which was incubated at 37 °C for 2 h so that the culture could attain its logarithmic growth phase. The number of viable cells (CFU) was then determined and trimeprazine was added at a concentration slightly higher than MIC of the respective strain. After every 2 h up to 6 h and then at 18th h CFU count was determined in the same culture.

Animal Protection Test. 50LD₅₀ challenge dose corresponding to 0.95×10⁹ CFU of *Salmonella typhimurium* 74 was suspended in 0.5 ml sterile peptone water and this dose was used for each mouse. The challenge test dose was reproducible as confirmed by measuring its optical density at 640 nm in a Klett Summerson colorimeter, which would give the predetermined number of CFU on nutrient agar. The animals were injected intraperitoneally 150, 75 or 40 µg/gm trimeprazine or 0.1 ml of saline (control), 3 h before the challenge. The survival of the trimeprazine-treated and control mice was recorded up to 100 h (Table II).

Determination of bacterial counts in organ homogenates of trimeprazine treated mice. All the mice used for this experiment received the challenge dose (which was the same as before); 50% of the population were given the drug (0.75 µg/gm of mouse) 3 h before the challenge while the remaining 50% received only saline. All the animals were autopsied 18 h after the challenge, their livers and spleens were removed, homogenised in a glass homogeniser and preserved at –20 °C for determination of CFU/ml. The homogenised livers and spleens were diluted tenfold in 4.5 ml sterile peptone water. Prior to inoculation, the plates containing solid medium were overdried at 37 °C to facilitate quick

absorption of the inoculum. The plates were divided into 6 sectors and each of the sectors received one drop of a corresponding dilution from a 50-dropper pipette.

Bacterial counts were determined in sectors showing the largest number of colonies without confluence. The mean of 6 counts was taken to determine the CFU per ml of the dilution [20, 21].

Results

In vitro antimicrobial action of trimeprazine. Trimeprazine proved to have powerful antimicrobial activity against both Gram positive and Gram negative bacteria. Table I shows that out of 23 strains of *Staphylococcus* spp., 19 strains were inhibited at 10–50 µg/ml of trimeprazine. Strains of *Bacillus* spp. were inhibited at 10–100 µg/ml. Seven out of 10 strains of *Salmonella* spp. were inhibited by 100 µg/ml. Trimeprazine could also inhibit 21 out of 26 strains of *Shigella* spp., 42 out of 54 strains of *Vibrio cholerae* and 18 out of 20 strains of *V. parahaemolyticus* at 10–100 µg/ml.

Strains of *Klebsiella* spp., *Proteus* spp., *Pseudomonas* spp. and *Citrobacter* were moderately sensitive to trimeprazine. Figure 1 shows the bactericidal action of 40 µg/ml trimeprazine on *S. aureus*.

In vivo experiments. Two strains of *S. typhimurium* were used, however, the results with respect to *S. typhimurium* 74 only is being presented (Table II).

Table I

Inhibitory spectrum of trimeprazine

Bacteria	No. inhibited by trimeprazine at (µg/ml)						
	Tested	10	25	50	100	200	400
<i>Staphylococcus aureus</i>	20	2	10	4	4		
<i>Staphylococcus</i> spp.	3		1	2			
<i>Bacillus</i> spp.	5	1		2	2		
<i>Salmonella typhi</i>	8	1			4	3	
<i>Salmonella typhimurium</i>	2	1			1		
<i>Shigella</i> spp.	26	5	6	5	5	5	
<i>Escherichia coli</i>	70					70	
<i>Klebsiella</i> spp.	7						7
<i>Proteus mirabilis</i>	6					6	
<i>Citrobacter freundii</i>	2					2	
<i>Vibrio cholerae</i>	57	7	15	15	5	8	4
<i>Vibrio parahaemolyticus</i>	20	9	8	7	2		
<i>Pseudomonas</i> spp.	20				8	5	7

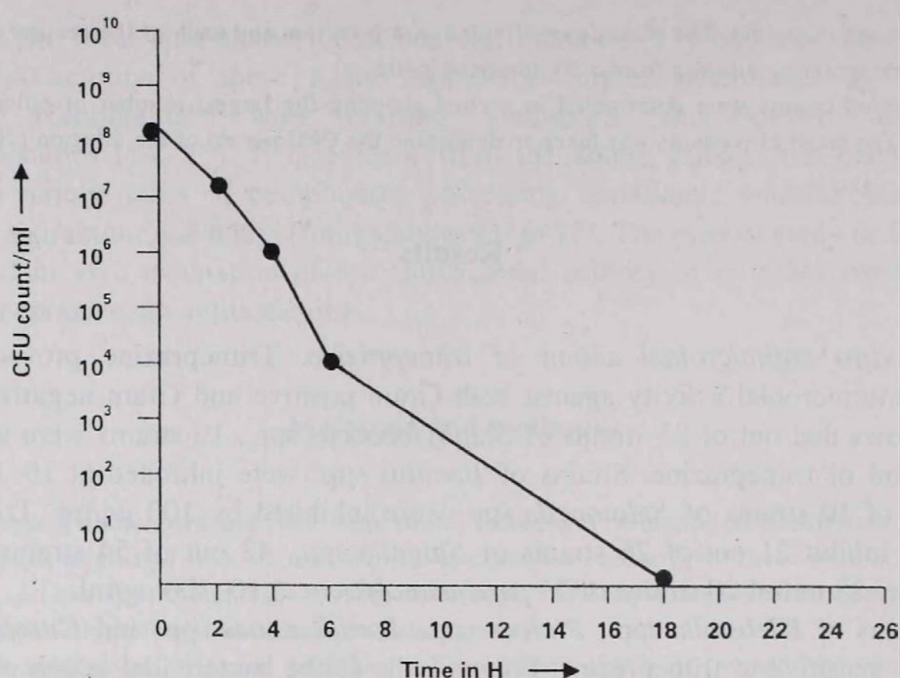


Fig. 1. The action of trimeprazine on *S. aureus* ML 275 (MIC 25 µg/ml)

Table II

Effect of trimeprazine on survival of mice challenged with Salmonella typhimurium NCTC 74

Treatment	Mice dying
Trimeprazine 0.75 µg/g	8 out of 20
Trimeprazine 0.4 µg/g	7 out of 20
Saline (control experiment)	49 out of 60

Infecting dose (0.95×10^9 CFU of *S. typhimurium* 74) was given 3 h after treatment

1.5 µg/g trimeprazine proved to be toxic. Table II shows that 0.75 µg/g and 0.4 µg/g trimeprazine given 3 h before the challenge reduce the mortality of mice. This effect proved to be significant according to χ^2 test.

In order to determine whether trimeprazine can reduce the bacterial counts in organ homogenates of challenged animals, 10 mice were divided into 2 groups. All the mice received a challenge dose of 0.95×10^9 CFU/ml of *S. typhimurium* 74. Five mice received 0.75 µg/g trimeprazine 3 h before the challenge, while the remaining 5 received only saline. The mice were autopsied 18 h after the challenge; homogenates of their liver

and spleen were prepared for viable counts. Table III shows that the viable counts of trimeprazine administered animals were considerably lower than that observed in mice which did not receive the drug. A similar protection by trimeprazine was observed when *S. typhimurium* 11 was used instead of *S. typhimurium* 74.

Discussion

During the past few years a large number of compounds belonging to different pharmacological groups have been studied for their antimicrobial activity [11–13] and phenothiazines have shown to be the most promising among these.

By in vitro and in vivo tests we have found that phenothiazine trimeprazine also possesses powerful antibacterial action since strains of *Staphylococcus*, *Shigella*, *Salmonella* and *Vibrio* spp. were remarkably sensitive to this agent. However, *E. coli*, *Proteus*, *Klebsiella* and *Citrobacter* were comparatively less sensitive to trimeprazine. The noteworthy finding that emerged from this study was that *Pseudomonas* spp. normally resistant to common antibiotics were susceptible to this compound.

Table III

Viable counts (CFU/ml) of *S. typhimurium* 74 in organ homogenates of trimeprazine treated mice

Time of sampling	Mouse No.	Drug ($\mu\text{g/g}$)	CFU/ml counts in	
			Liver	Spleen
18 hour	1	Trimeprazine (0.75)	1.1×10^7	2.1×10^7
	2		1.8×10^7	3.9×10^7
	3		2.7×10^7	8.2×10^7
	4		3.5×10^7	4.4×10^7
	5		4.0×10^7	6.0×10^7
18 hour	1	Saline (control)	0.31×10^9	1.5×10^9
	2		1.0×10^9	0.9×10^9
	3		1.9×10^9	1.2×10^9
	4		1.9×10^9	1.1×10^9
	5		0.3×10^9	1.3×10^9

Statistical analysis of counts of 18 h liver and spleen homogenates was performed by *t*-test

A major mechanism of action of various phenothiazines, including trimeprazine derivatives, appears to be the membrane destabilising effects allowing intracellular drug-entry; this probably results also in anti-pili, anti-conjugative, antiplasmid (by intercalation

into DNA and inhibition of DNA gyrase) and overall antibacterial and anti-tumour actions [4, 16–18, 23].

Thus, this study on trimeprazine further opens up a new field of hitherto unsuspected class of chemotherapeutic agents, with unconventional antimicrobial properties, which will hopefully give rise to a new approach towards successful clinical chemotherapy.

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EFFECT OF BENOMYL ON *SACCHAROMYCES CEREVISIAE* DURING CONTINUOUS CULTIVATION

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The effect of Benomyl on *Saccharomyces cerevisiae* during continuous cultivation was investigated. The RNA, total protein and phosphorous contents decreased while the trehalose content increased with increasing Benomyl concentrations when an RD mutant and the parent strain were cultivated at different Benomyl concentrations under continuous conditions. The specific oxygen uptake rate of the respiratory deficient mutant (RD mutant) was approximately 5 times lower in comparison to the parent strain. The RQ value for the RD mutant was higher than 1 which clearly indicated the anaerobic metabolism of glucose degradation. The RD mutant lacked cytochrome *aa₃*, while the cytochromes *a* and *b* contents were lower than those of the parent strain.

Saccharomyces cerevisiae is an important microorganism in the industry [1], which is used often as a model microorganism for studies of eukaryotic cells. Special attention has been paid to the effect of different compounds and elements during the cultivation of *S. cerevisiae* in media containing molasses [2, 3]. The results showed that some compounds and elements inhibited the yeast growth completely. It is well known that a great number of chemical compounds may be genotoxic to *S. cerevisiae* [7]. Among them pesticides have deserved a special interest. The degradation products of pesticides used for sugar beet protection enter the molasses and may inhibit the yeast growth. Zauner et al. [8] investigated how the respiration intensity of *S. cerevisiae* cells depended on the concentration of different pesticides in molasses. The results showed that the residual pesticide concentrations in molasses were in the range of 0.05 to 50 ppm, and the pesticides affected negatively the respiration intensity of *S. cerevisiae*. Fatichenti et al. [9] investigated if the degradation of residual pesticides in *S. cerevisiae* fermentations was influenced by the metabolism of yeast. Six products used commonly in viniculture were tested. During the fermentations, some pesticides remained unaffected, while some others were degraded in a non-enzymatic way and some were metabolised by the yeast. Delgado and Conde [10] reported that Benomyl, a commercial fungicide which is known as an

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inhibitor of fungal microtubule polymerization [11], prevented mating – mediated nuclear fusion in *S. cerevisiae*. Mating mixtures prepared in the presence of Benomyl showed normal occurrence of cell fusion but a dramatic concomitant reduction in the frequency of nuclear fusion was observed. Benomyl induced frequently mitotic losses of chromosomes during the vegetative growth of *S. cerevisiae* [12]. Using *S. cerevisiae* D 61. M (mitotic chromosomal malsegregation assay), Benomyl showed a dose-dependent increase in the frequency of chromosomal malsegregation with the lowest effective dose tested of 0.1 mM [13]. The Benomyl and Benomyl + pirimiphos-methyl mixture did not show any genotoxic activity [14].

Here we present the effect of Benomyl on the composition of the cells, respiration intensity and cytochromes content in a parent and petit mutant strain of *S. cerevisiae* under continuous cultivation.

Materials and methods

Microorganism and inoculum preparation. Pure cultures of *S. cerevisiae* 163 parent strain and *S. cerevisiae* 14/I petit mutant, a descendant of *S. cerevisiae* 163, were used [15, 16]. Both microorganisms were from the collection of the Faculty of Technology of the University of Novi Sad. The pure cultures were maintained on slants using culture medium which consisted of glucose 20 g/l, peptone 10 g/l, yeast extract 5 g/l, agar 20 g/l [17]. The cultures were stored at 4 °C following incubations at 30 °C for 48 h. During inoculum preparations loopful slant cultures were transferred to 50 ml aliquots of van der Walt medium in 250 ml culture flasks [17] then incubated in an orbital shaker (200 rpm) at 30 °C for 24 h.

Fermentation medium. The medium consisted of glucose 20 g/l, peptone 10 g/l, yeast extract 5 g/l. The pH was adjusted to 5.0. Sterilization was carried out at 121 °C for 30 min. After sterilization Benomyl was added to the media under sterile conditions. The Benomyl concentrations tested were as follows: 3.0, 6.0, 9.0 and 12 mg/l. Benomyl stock solutions were prepared by dissolving it in 5 ml of 96% ethanol.

Equipment and fermentation. Fermentation was carried out in a 10 liter fermenter (Chemap, Volketswil, Switzerland) consisting of a top-driver stirrer, a water-cooled condenser on the air outlet, and built-in foam, temperature, pH and dissolved oxygen (DO) control systems. Dissolved oxygen and pH were measured with a model 900 DO probe (New Brunswick NI, USA) and with an Ingold (Zürich, Switzerland) pH electrode, respectively. The working volume was 5.0 liter, and a good mixing was assured by two flat-blade agitators set at 10 Hz. The air flow rate was 240 l/h, and temperature was maintained at 30 °C. The fermenter was equipped with an additional unit that measured the biomass (liquid) in the system, whereas the substrate concentrations were kept constant (chemostat). The in- and outflowing air streams were continuously monitored for oxygen and carbon dioxide concentrations using a gas analyzer (Hartmann and Braun, Frankfurt/Main, Germany). During cultivations the flow of culture media was regulated by peristaltic pumps to reach and maintain the selected dilution rate (D).

Chemicals. Benomyl (methyl-1-(butyl carbamoyl)-2-benzimidazole-carbamate) was obtained from Du Pont Co. All chemicals were of analytical grade or the highest purity available.

Analytical methods. Samples were taken for the determination of dry matter [18], cytochromes [19], trehalose [20], nitrogen and phosphorus [21] and ribonucleic acids [22].

The specific oxygen uptake and the specific carbon dioxide production rate, as well as the respiration quotient were calculated [23].

Results and discussion

The effects of Benomyl concentrations on the composition of the cells during continuous cultivations are summarised in Table I.

Comparing the RD mutant and the parent strain, a significant difference was observed in the protein, phosphorous and RNA content. Increasing Benomyl concentrations resulted in decreasing protein and phosphorous contents of the cells of both the parent and the RD mutant strain. These results are in accordance with previous data [24]. The parallel decrease of the nitrogen and phosphorus content of the cells could be explained well assuming that the cells were trying to maintain a constant nitrogen-to-phosphorus ratio which is a well-known characteristic of *S. cerevisiae* [25]. The trehalose content increased with increasing of Benomyl concentrations (Table I). Trehalose is a storage disaccharide, which is a potential factor in stress tolerance of yeast. Previous studies demonstrated that this sugar accumulated in *S. cerevisiae* [26, 27] and *Schizosaccharomyces pombe* [28] during exposure to noxious agents, indicating that it might play a role in the inducible stress response of yeasts. Stress tolerance of *S. cerevisiae* varies considerably with the physiological state and the cultural conditions [29]. For example, cells with exponential growth phase are more sensitive to stress caused by physical and chemical mutagens [30] than cells that are growing more slowly or are resting.

Table I

Effect of Benomyl concentrations* on the cell composition of *Saccharomyces cerevisiae* 163 parent and an RD** mutant 14/1 strain. Dilution rate $D=0.2\text{ h}^{-1}$

Benomyl mg/l***	Dry matter in yeast sample %		Proteins % of dry matter		Phosphorous % of dry matter		Trehalose % of dry matter		Total ribonucleic acids % of dry matter	
	parent strain	RD*	parent strain	RD*	parent strain	RD	parent strain	RD	parent strain	RD
0	26.5	26.1	52.40	43.80	2.8	2.3	0.35	1.20	6.98	4.90
3	26.3	25.8	52.05	43.10	2.7	2.1	0.88	1.75	6.10	4.27
6	25.9	25.5	51.58	42.88	2.5	2.0	1.79	2.52	5.50	3.85
9	25.8	25.2	49.14	42.10	2.3	1.9	2.56	3.96	4.65	3.28
12	25.5	24.9	48.40	41.25	2.0	1.7	3.70	4.40	4.08	2.87

* Mean values of five exchange of fermenter content with the same Benomyl concentrations

** RD - respiratory deficient mutant

*** Benomyl concentration at the new steady state in fermenter

Specific oxygen uptake rates (Q_{O_2}), specific CO_2 production rates (Q_{CO_2}) and respiration quotients (RQ) are shown in Figs 1 and 2, for the *S. cerevisiae* parent strain and the RD mutant, respectively. The specific oxygen uptake and the specific carbon dioxide release were adversely related to the Benomyl concentrations in the parent strain. The decrease in the RQ values indicated that oxidative glucose degradation was intensified (Fig. 1). On the other hand, the specific oxygen uptake of RD mutant cells, which were cultivated under the same experimental conditions as the parent strain cells, was approximately 5 times lower compared to the parent strain. This could be clearly attributed to the loss or severe damage of the respiratory chain in the RD mutant. After addition of Benomyl both the Q_{CO_2} and RQ values increased while the specific oxygen uptake rates decreased further (Fig. 2). This means that glucose degradation developed further towards anaerobic metabolism.

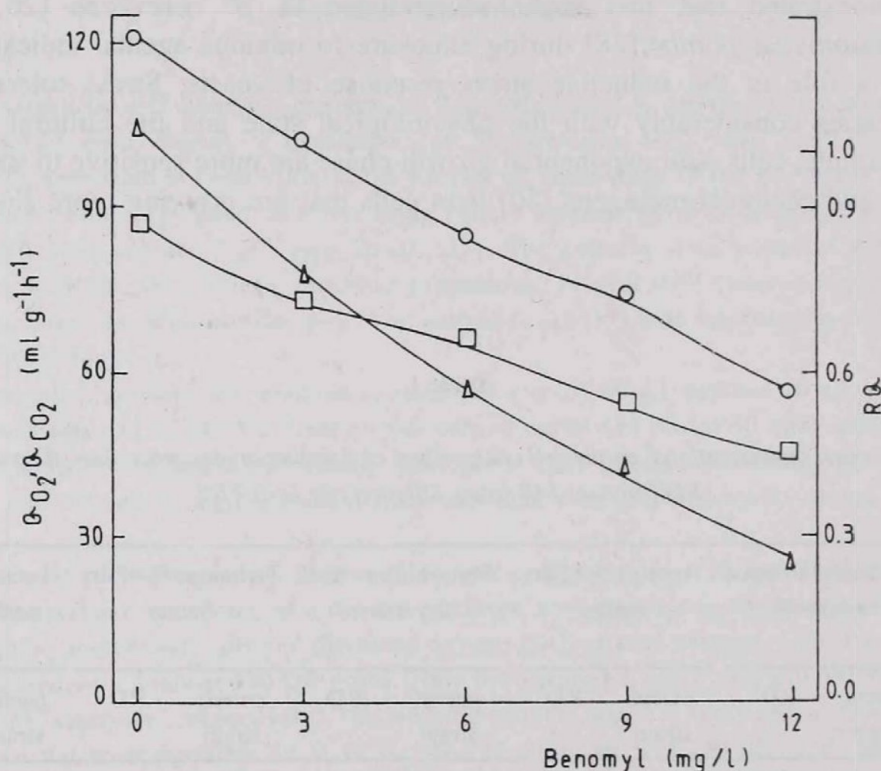


Fig. 1. Dependence of the specific oxygen uptake rate Q_{O_2} (○), specific CO_2 production rate Q_{CO_2} (△) and respiration quotient RQ (□) on the Benomyl concentrations in *Saccharomyces cerevisiae* 163 parent strain. Dilution rate $D=0.2\text{ h}^{-1}$

Cytochromes have been divided into four groups according to the chemical structure of the heme prosthetic group. Each of the four heme types, a, b, c and d, has a different side chain on its porphyrins [31].

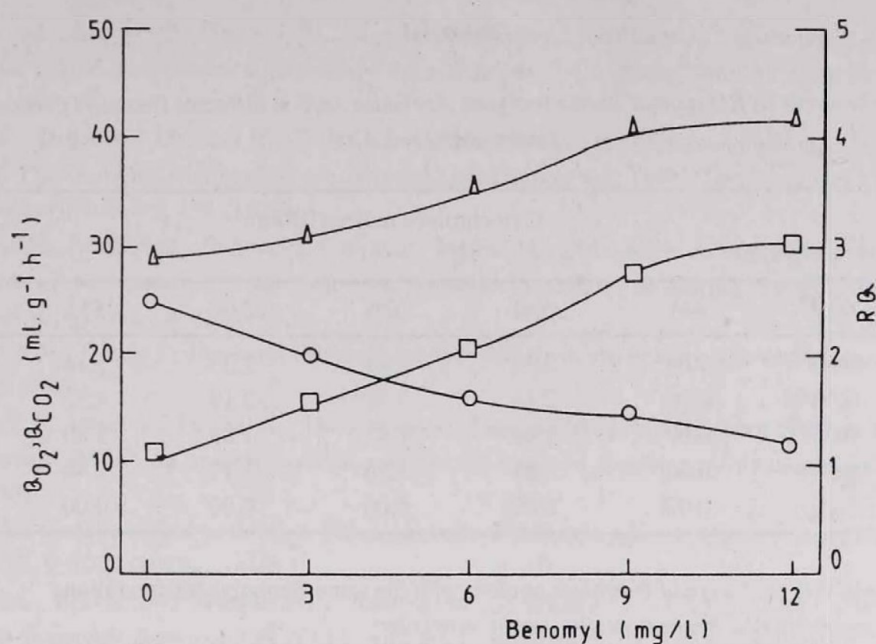


Fig. 2. Dependence of the specific oxygen uptake rate Q_{O_2} (○), specific CO_2 production rate Q_{CO_2} (Δ) and respiratory quotient RQ (□) on the Benomyl concentrations in RD mutant 14/l *Saccharomyces cerevisiae*.

Dilution rate $D=0.2\text{ h}^{-1}$

Table II

Cytochrome contents in parent strain *Saccharomyces cerevisiae* 163 at different Benomyl concentrations*.

Dilution rate $D=0.2\text{ h}^{-1}$

Benomyl (mg/l)**	Cytochromes moles×10 ⁵ /dm ³						Total
	aa ₃		b		c		
	605***	444	550	520	560	532	
0	0.68	1.02	2.96	4.67	2.75	6.77	18.85
3	0.32	0.65	2.51	3.81	2.54	5.86	15.69
6	0.14	0.88	2.43	3.64	2.18	3.98	13.25
9	0.09	1.05	2.32	2.83	2.03	3.57	11.89
12	0.00	0.63	1.01	1.65	0.80	1.35	4.87

* Mean values of five exchange of fermenter content with the same Benomyl concentrations

** Benomyl concentration at the new steady state in fermenter

*** Wavelength (nm)

Table III

Cytochrome contents in RD mutant Saccharomyces cerevisiae 14/1 at different Benomyl concentrations.
Dilution rate $D=0.2\text{ h}^{-1}$*

Benomyl (mg/l)**	Cytochromes moles×10 ⁵ /dm ³						Total
	aa3		b		c		
	605***	444	550	520	560	532	
0	0.00	0.00	2.62	3.84	2.35	5.14	13.95
3	0.00	0.00	2.14	3.52	2.19	4.32	12.17
6	0.00	0.00	1.98	1.85	1.89	3.80	9.52
9	0.00	0.00	1.82	1.56	1.77	2.40	7.55
12	0.00	0.00	0.00	0.00	0.00	0.00	0.00

* Mean values of five exchange of fermenter content with the same Benomyl concentrations

** Benomyl concentration at the new steady state in fermenter

*** Wavelength (nm)

The cytochrome content of the *S. cerevisiae* parent strain and the RD mutant cells are presented in Tables II and III. Cytochromes aa₃, b and c were found in *S. cerevisiae* parent strain cells (Table II), while the RD mutant cells contained lower quantities of cytochromes b and c. Moreover cytochrome aa₃ was not found in RD mutant at all. Increasing Benomyl concentrations resulted in decreasing cytochrome contents during continuous cultivation of *S. cerevisiae* parent strain and RD mutant. These results are in accordance with Oura's suggestion [32] that cytochrome contents may depend on cultivation conditions. However, the presence or absence of cytochromes may be used for the estimation of the possible genotoxic effect of different chemicals.

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DETECTION OF VTEC USING SPECIFIC DNA PROBES AND COMPLEX TYPING OF *ESCHERICHIA COLI* O157

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The incidence of *E. coli* causing hemorrhagic colitis (HC) or non-bloody enteritis in Hungary was studied using SLT-I and SLT-II gene-probes as well as Vero-cell toxicity and Verotox-F tests. Out of 41 *E. coli* O157 strains isolated in Hungary between 1987 and 1996 15 strains (O157:HNM 4, O157:H7 8, O157:HNT 3) derived from hemorrhagic colitis (HC). Hybridization was observed with SLT-I and/or SLT-II in 19 strains. Verocytotoxin production of *E. coli* of 23 other serotypes was proven by hybridization of DNA probes. SLT production were demonstrated in 24 strains. Complex typing (sero-, phage-, colicin-typing and plasmid profile analysis) was carried out in *E. coli* serogroup O157 strains isolated from different geographical areas. Using the Hungarian phages the *E. coli* O157:HNM, O157:H7 strains could be distributed into 6 phage groups each and these phage groups could be further divided according to colicin production and plasmid profile. The Hungarian phage typing method for *E. coli* strains used since 1978 was compared to the method elaborated in Canada in 1990. Out of the most frequent Canadian phage types (1, 4, 8, 31, 14) phage types 8, 31 and 14 were observed in Hungary.

In the last 15 years the attention was called to a new enteropathogenic group of enterohemorrhagic *E. coli* (EHEC) producing verocytotoxins (VT) or so-called Shiga-like toxins (SLT) by wide spread epidemics with unusual symptoms and serious outcome. The first extended epidemics were reported from the USA [1, 2] but epidemics caused by these pathogens were also observed in other continents and in some European countries afterwards [3–7]. The systematic examinations on the incidence of the VT producer strains belonging to serogroup O157 started in Hungary in 1987, and the results have been published by Tóth et al. [8], Herpay et al. [9–11], Czirók and Herpay [12], Milch et al. [13, 14].

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Materials and methods

Bacterial strains: Out of 442 *E. coli* strains examined for VT production between 1987 and 1996, 133 strains belonged to serogroup O157 of different serotypes and 70 strains belonged to 23 other serotypes. The origin of human strains was enteritis (E), hemorrhagic colitis (HC), and the animal strains were isolated from cows, pigs and meat products.

Strains examined by complex typing: Out of 129 *E. coli* O157 strains examined by complex typing, 17 strains of O157:NM were isolated in the UK, USA, Germany, and Hungary, 86 O157:H7 strains were isolated in the USA, Canada, Germany, Kuwait and Hungary. These strains originated from human cases, either with hemorrhagic colitis (HC), or with hemolytic uremic syndrome (HUS), as well as from cows and pigs. The 26 *E. coli* O157 strains of 9 different other serotypes: O157:H2, O157:H3, O157:H16, O157:H19, O157:H33, O157:H38, O157:H39, O157:H42, O157:H45 and O157:HNT were isolated in Germany, Ireland, Russia and Hungary.

Media culture condition and serotyping of strains was described previously [11].

Vero-cell toxicity test. For VT detection the methods of Konowalchuk et al. [15] was carried out.

Verotox-F test product of Denka Seiken Co Ltd., Tokyo Japan was used.

DNA probes. The recombinant pJN37-19 and pNN111-19 plasmids containing the SLT-I and SLT-II probes were kindly provided by J.W. Newland (Walter Reed Army Institute of Research, Washington D.C. [16]. Table I shows the data of the DNA probes.

O'Brien et al. [17] established that the terms VT and SLT were synonymous, so our results were given on the basis of hybridization with SLT-I and SLT-II probes.

Isolation of plasmid DNA was performed according to Kado and Liu [18]. The restriction enzymes Bam I and Pst I were used as described by the producer (Amersham International, UK). The SLT-I and SLT-II probe specific fragments were isolated as described by Table I. The probe specific fragments were extracted from the agarose using the GeneClean kit (Bio 101 Inc, La Jolla, CA, USA) according to the manufacturer's description.

DNA labelling and colony hybridization. Probes were labelled by ^{32}P , using a random priming method and Megaprime kit (Amersham). Hybond-N nylon hybridization membranes (Amersham) were placed onto colonies grown on McConkey agar. After 1 h blotting at room temperature, the blotting paper was saturated with 0.5 M NaOH and 1.5 M NaCl for 1 min, followed by 1 min heating at 100 °C for further lysis. Samples were neutralized in a mixture of 1 M Tris (pH 7.2) and with 2 M NaCl. Filters were dried at room temperature. The colony hybridization by ^{32}P labelled DNA probes and the autoradiography was performed according to Baldini et al. [19]. After hybridization the unbound probe was removed then the membranes were packed into cellophane with X-ray film in an X-ray cassette. The films were exposed depending on the activity determined after the last washing of the membranes (−70 °C, 1–7 days, in case of higher activity: room temperature).

Complex typing:

– **Serotyping:** determination of O and H antigen was carried out according to Orskov and Orskov [20], modified as described previously [21].

– **Phage typing.** The method described by Milch and Gyenes [22] and Milch [23], for comparison the method of Khakhria et al. [24] were used.

– **Colicin typing** was carried out according to Lafont et al. [25] and Horak [26].

– **Plasmid profile analysis.** Plasmid DNA was prepared according to Kado and Liu [18]. Agarose gel electrophoresis was performed in vertical agarose gel according to Meyers et al. [27].

Plasmid standards used to determine molecular weight. *E. coli* V517 [28]; 1.4, 1.8, 2.0, 2.6, 3.4, 3.7, 4.8, 35.8 Md, R1: 62 Md, R27: 112 Md, pSp1: 140 Md.

Table I

DNA probes for hybridization

DNA probe	Plasmid	Fragment	Restriction enzyme	Original plasmid phage	Vector	Origin
SLT-I	pJN 37-19	1142 bp	Bam III	933 J (φ)	pUC19	Newland J.W. (16)
SLT-II	pNN 111-19	842 bp	Pst I	933 W (φ)	pUC18	Newland J.W. (16)

Table II

VT production of E. coli O157 strains isolated in Hungary

Serotype	Cytopatho- genic effect ^x	F-test		DNA probe		Diagnosis	Origin	No. of strains
		V T-1	VT-2	SLT-I	SLT-II			
O157:HNM	-	-	-	-	-	E	Pig	6
O157:HNM	+	+	+	+++	+++	HC	Pest county	3
		+	-	+	-	HC	Vas county	1
O157:H7	+	+	+	+	+++	HC	Budapest	8*
	.	-	++	-	++	E	Somogy county	1
O157:H2	-	-	-	-	-	E	Budapest	3
O157:H3	-	-	-	-	-	E	Veszprém	1
O157:H16	-	-	-	-	-	E	Somogy county	1
	.	-	-	-	-	E	Zala county	1
	.	-	-	-	-	E	Veszprém	2
O157:H33	-	-	-	-	-	E	Imported case	1
O157:H38	-	-	-	-	-	Pus	Somogy county	1
O157:H39	-	-	-	-	-	E	Budapest	1
O157:H45	-	-	-	-	-	E	Budapest	1
O157:HNT	+	+	+	+	+	HC	Budapest	3
	-	-	-	-	-	E	Veszprém	1
	-	-	-	-	++	E	Veszprém	1
	-	-	-	-	+	E	Borsod county	1
	-	-	-	-	-	Sl	Budapest	1
	.	+	+	++	++	E ^{xx}	Budapest	1
	-	-	-	-	-	E	Budapest	1
	-	-	-	-	-	E	Budapest	1
Total								41

^xon Vero-cell line

HC = hemorrhagic colitis

^{xx}food poisoning

E = enteritis

*from one patient

Sl = symptomless

. not done

Results

Results of DNA hybridization and cytotoxin production. Table II shows the cytotoxicity by Vero-cells and VT production by F-test and results of DNA probes of 41 *E. coli* strains of serogroup O157 belonging to different serotypes, isolated in Hungary between 1987 and 1996. VT production were demonstrated by F-test in serotypes O157:HNM, O157:H7 and O157:HNT (17 strains). The results obtained with F-test and DNA probes were identical except of two strains: the two O157:HNT strains were negative with F-test, but they were positive with SLT-II DNA probes.

Table III

Other VTEC serogroups than O157

Serotype	SLT-I	SLT-II	SLT-I	SLT-II	Diagnosis, origin	Total
O1:H6	—	—	1	—	E	1/1
O4:H5	—	—	—	3	E	3/8
O6:HNM	1	—	—	—	HC	1/1
O6:H16	1	—	—	—	E	1/1
O6:HNT	1	—	—	—	HC	1/1
O7:H12	—	—	—	2	E	2/2
O9:H19	—	—	—	1	HUS	1/1
O26:HNM	4	—	—	1	E	5/9
O26:H1	1	—	—	—	E	1/1
O26:H11	1	—	—	3	E	4/10
O26:HNT	1	—	—	—	E	1/5
O39:H48	—	—	1	—	Cow	1/1
O45:HNT	—	—	—	1	Beef	1/1
O55:H32	1	—	—	—	HC	1/1
O86:H18	—	—	—	1	E	1/1
O111:HNM	5	—	—	—	HC (1) E (4)	5/6
O111:H12	3	—	—	2	E	5/7
O111:H31	1	—	—	—	E	1/1
O111:HNT	1	—	1	—	E	2/2
O114:HNT	—	—	—	1	HC	1/1
O128:H12	—	—	—	6	Milk (5) HC (1)	6/6
O139:H21	—	—	—	2	E	2/2
O141:H8	—	—	—	1	Food poisoning	1/1
	21	—	3	24		48/70

HC = hemorrhagic colitis

HUS = hemolytic uraemic syndrome

Hybridization of 70 *E. coli* strains belonging to other (not O157) serogroups and to different serotypes was performed with SLT-I and SLT-II probes as well. Verocytotoxin production was found in 48 strains. Hybridization was observed with SLT-I and SLT-II probes in case of 21 strains, with SLT-I probe in 3 strains. Genes determining only SLT-II toxin production were demonstrated in the majority of the verocytotoxin producer strains (24 strains) belonging to 16 different serogroups and 23 serotypes (Table III).

Results of complex typing of O157:HNM strains. Using the Hungarian phages the 20 *E. coli* O157:HNM strains could be distributed into six groups. Strains of serotype O157:HNM isolated in the USA belonged to three different phage groups and plasmid profiles. Phage groups and plasmid profiles of the strains isolated in UK and Hungary differed from the phage groups and plasmid profiles of the USA strains.

Colicin was produced by 6 strains originated from pigs and plasmid of size 65 Md was carried by 10 human strains. Within one phage group 1 or 3 kinds of plasmid profiles were found (Table IV).

Table IV

Complex typing of E. coli O157:HNM strains of different origin

Phage group	Colicin type	Plasmid profile (Md)	Location	Origin, diagnosis	No. of strains
A1	–	73 57	UK	Pig, E	1
A1 A2	–	65 2.2	USA	Human, HC	2
A1 A2 A3	–	65 4.2 2.2	USA	Human, HC	1
	–	65 2.2	Germany	Human, HC	2**
	–	65 26	USA	Human, HC	2
A1 A2 A3 A4	–	65 4.2 2.2	USA	Human, HC	1
		60	Hungary	Human, HC	2**
	–	65	Hungary	Human, HC	1**
A1 A2 A3 B1	–	65 1.4	Hungary	Human, HC	1**
NT	E11a	140 100 85 67	Hungary	Pig, E	2*
		4.6 3.6 2.3			
		90 77 37 4.3	Hungary	Pig, E	2*
		3.7 2.5			
		77 42 6.5 4.9 4.2	Hungary	Pig, E	2*

NT = non-typable

* = SLT-I and SLT-II negative

** = SLT-I and SLT-II positive

The 86 *E. coli* strains of serotype O157:H7 belonged to 6 different phage groups. The most frequent phage groups were A1 A2 A3 and A1 A2 A3 A4 which could be divided according to plasmid profile into 14 each. The most frequent plasmid profiles were: 65, 2.2; 65; 65, 4.2, 2.2; 65, 4.2; 65, 5.4 4.2, 2.2. Strains of serotype O157:H7 isolated in the USA and Canada showed 6-kinds of phage groups and 18-kinds of plasmid profiles. The strains isolated in Germany could be grouped in four different phage groups and plasmid profiles. The phage groups of the eight strains isolated in Hungary were identical with the most frequent phage groups of the USA strains but their plasmid profile differed. Phage groups of O157 strains with H antigens different from HNM or H7 differed – except of two – from the H7 and NM strains and seven strains had a plasmid profile identical with them.

Out of the 86 O157:H7 strains 11 were colicinogenic; the colicin types were: D, E₆Ib and non characteristic (Table V).

Table V

Complex typing of E. coli O157:H7 strains of different origin

Phage group	Colicin type	Plasmid profile (Md)	Location	Origin, diagnosis	No. of strains
A1 A2	–	2.2	USA	Human HC	1
	–	65 35.8 7.0	Germany	Human HC	1
A1 A3	–	65 30 4.2 2.2	USA	Human HC	1
A1 A2 A3	–	65 22 2.2	USA	Human HC	5
	D	65 22 4.2	USA	Human HC	1
	–	65 22	USA	Cow	3
	–	65 5.4 4.2	USA	Human HC	4
	–	65 4.2 2.2	USA	Human HC	5
	–	65 2.2	USA	Human HC	3
	–	65	USA	Human HC	3
	D	65 4.2	USA	Human HC	5
	–	65 55 26 20 4.2 2.2	USA	Human HC	1
	D	65 52 4.2	USA	Human HC	1
	–	65 50 2.3	Hungary	Human* HC	8**
	–	65 34	USA	Human HUS	1
	–	65 34 30	Hungary	Human HC	1***
	E ₆ Ib	70 3.9	Germany	Human HC	1**
A1 A2 A3 A4	–	65	USA (3) Kuwait (3)	Human HUS	6****
	–	2.2	USA	Human HC	3

Continued on p. 79.

Table V continued

	-	65 2.2	USA	Human HC	5
	-	65 4.2 2.2	USA	Human HC	3
	-	65 3.4 2.2	USA	Human HC	1
	NC	65 4.2	USA	Cow	1
	D	65 4.2	USA	Human HUS	1
			Canada	Human HC	1
			Germany	Human HC	1**
	-	65 5.4 4.2 2.2	USA	Human HC	4
	-	65 22	USA	Cow	3
	-	65 22 4.2 2.2	USA	Human HUS	1
	-	65 22 4.2 3.5 2.2	USA	Human HC	2
	-	65 5.4 4.2 2.2	USA	Human HC	2
	-	65 42	USA	Human HC	1
	-	65 57	USA	Human HUS	1
A1 A2 A4 B1	-	65 2.2	USA	Human HC	1
	-	4.2 2.2	USA	Mutant	1
A1 A2 A3 A4 B1	-	65 2.2	USA	Human HC	1
	-	65	USA	Human HUS	1
E6Ib		4.2 3.9	Germany	Human HUS	1**
Total					86

SLT-I and SLT-II hybridization were done only by strains from Hungary, Germany and Kuwait

NC = non-characteristic

* = one patient

** = SLT-I and SLT-II positive

*** = SLT-II positive

**** = SLT-I and SLT-II positive (5)

SLT-I negative SLT-II positive (Kuwait 1)

Among 27 strains of *E. coli* O157 belonging to serotypes other than O157:HNM or O157:H7 ten-kinds of H antigens, different from H7 were found: H2, H3, H16, H19, H33, H38, H39, H42, H45, HNT. These strains originated from HC, HUS, nonbloody diarrhea and pus. Seven strains showed identical phage group with the strains of antigen H7 or HNM. Plasmid profiles of the O157 strains having various H antigens were heterogeneous. Out of the 26 strains 9 carried a plasmid of 65 Md and five strains were colicinogenic (Table VI).

Khakhria et al. [24] elaborated in Canada a phage typing method exclusively for the *E. coli* O157:H7 and O157:HNM strains. The comparison of our phage typing scheme with the Canadian typing method is demonstrated on the Table VII a, b, c. In the case of O157:HNM and O157:H7 strains the Canadian method gives a better possibility for the differentiation but in case of strains possessing H antigen other than H7 the Hungarian phages were more effective.

Table VI

E. coli O157 strains belonging to serotype other than O157:H7 or O157:HNM

Serotype	Phage group	Colicin type	Plasmid profile (Md)	Location	Origin, diagnosis	No. of strains
O157:H2	A3	—	Plasmidless	Hungary	Human E	3
O157:H3	A1A2	—	8.5	Hungary	Human E	1
O157:H16	A2	NC	90 42 4.8	Hungary	Human E	1
	A2	NC	90 35.8 4.2 3.4 2.4	Hungary	Human E	1
	B2	V+NC	120 100 48 4.2	Hungary	Human E	1
O157:H19	B2	NC	65	Ireland	Pig E	1
O157:H33	A4	—	65 3.6 2.8 2.2	Russia	Human E	1
O157:H38	A1B2	—	90	Hungary	Human Pus	1
O157:H39	A1A2	—	1.6	Hungary	Human E	1
	A2	—	.	Germany	Human E	1
O157:H42	A3	—	65 2.0	Germany	Human E	1
O157:H45	B2	—	90	Hungary	Human E	1
O157:HNT	A2	—	65 50 46 32	Hungary	Human E	1
	A2	V	90 77 46	Hungary	Human E	1
	A1A3	—	65 2.2	Hungary	Human E	2
	A1A3	—	65 2.2	Hungary	Human HC	3*
	A1A3	E6Ib	4.2	Germany	Human HUS	1*
	NT	—	112	Hungary	Human FP	1*
	NT	—	.	Hungary	Human E	3
	NT	V+NC	75 62 2.7 1.8 1.4	Hungary	Human E	1

NT = non-typable;

E = enteritis

NC = non characteristic;

FP = food poisoning

* = SLT-I and SLT-II positive;

. = not done

Discussion

By the use of serotyping and DNA probes efforts were made to determine the incidence of *E. coli* O157 strains causing hemorrhagic colitis or non-bloody diarrhea in Hungary. Out of 41 *E. coli* O157 strains isolated in Hungary between 1987 and 1996 15 strains derived from HC cases, among these four strains belonged to serotype O157:HNM, 8 to O157:H7 (isolated from the same patient) and three strains were of serotype O157:HNT. Altogether 19 strains were of O157:HNM or O157:H7, 22 strains possessed other different H antigens. With SLT-I or SLT-II probes in all strains derived from HC, VT production was demonstrated. Hybridization with SLT-I and SLT-II probes was demonstrated only in case of one strain derived from enteritis case (food poisoning)

(O157:HNT). VT production was demonstrated only with hybridization (SLT-II probe) in two strains originated from enteritis. Hybridization results were supported by the biologic test and F-test, except in two cases.

It was reported [29–38], that VT production was demonstrated also in other *E. coli* serogroups than O157 and from strains belonging to other genus, therefore 70 *E. coli* strains belonging to other serogroups and other serotypes were examined. Out of the examined 63 human and 7 animal strains the hybridization was positive in 41 human and 7 animal strains. Among the SLT positive strains only 7 strains derived from HC or HUS (Table III). These data indicate that it is important to examine the VT production of *E. coli* serotypes derived from enteritis cases (especially in epidemics) and not only from HC.

Table VII.a

Comparison of the Hungarian and Canadian typing schemes

Hungarian phage group	No. of strains	Canadian phage type () No. of strains	Origin
O157:HNM			
A1	1	NC(1)	UK Pig
A1A2	2	31(1) 32(1)	USA Human
A1A2A3	5	14(1) 32(3) 34(1)	USA, Germany Human
A1A2A3A4	2	32(1) 8(1)	USA, Hungary Human
A1A2A3B1	1	14(1)	Hungary Human
NT	6	NT(6)	Hungary Pig
Total	17		

Strains isolated from outbreaks were examined only from the USA; only three of the strains originating from the seven outbreaks were homogeneous according to their phage groups but their plasmid profiles showed differences.

The comparison of the Hungarian phage typing method to the method elaborated in Canada resulted that the strains belonging to O157:H7 and O157:HNM were typable more effectively by the Canadian phages. The strains of other H antigen could be typed better with the Hungarian phages. Out of the most frequent Canadian phage types: 1, 4, 8, 31, 14 the phage types 8, 31 and 14 was observed in Hungary, too.

According to Canadian and USA authors [25, 40, 41] phage typing of *E. coli* O157:H7 strains provide more useful epidemiological informations than plasmid profile analysis. Our examinations proved that the differentiation by complex typing of *E. coli* O157 strains of different serotypes might be more reliable than only one of the typing methods.

Table VII.b

Hungarian phage group	No. of strains	Canadian phage type () No. of strains	Origin
O157:H7			
A1A2	1	32(1)	USA Human
A1A3	1	31(1)	USA Human
A1A2A3	40	1(4) 14(9) 21(5) 23(1) 31(8) 32(3) 34(7) 45(1) NC(1) NT(1))	Hungary Human Germany Human
A1A2A3A4	28	1(6) 14(5) 21(2) 23(1) 31(1) 32(5) 33(1) 34(5) NC(2)	USA Human, Cow, Meat Canada Human
A1A2A3A4B1	2	1(1) NC(1)	USA Human
Total	72		

Table VII.c

Serotype	Hungarian phage group	No. of strains	Canadian phage type () No. of strains	Origin
O157:H2	A3	3	NC(3)	Hungary Human
H3	A1A2	1	NC(1)	Hungary Human
H16	A2	3	33(2), NC(1)	Hungary Human
H19	B2	1	NT(1)	Ireland Pig
H33	A4	1	NC(1)	Russia Human
H38	A1B2	1	NC(1)	Hungary Human
H39	A1A2	1	NC(1)	Hungary Human
H39	A2	1	NC(1)	Germany Human
H42	A3	1	NC(1)	Germany Human
H45	B2	1	NT(1)	Hungary Human
HNT	(A1)A3	6	NC(6)	Hungary Human
	A2	2	NT(1), NC(1)	Hungary Human
	NT	5	NT(3), NC(2)	Hungary Human
Total		27		

NC = non-characteristic

NT = non-typable

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EFFECTS OF NIMODIPINE ON THE CEREBROVASCULAR AND NEURONAL CHANGES DURING PNEUMOCOCCAL MENINGITIS IN THE RAT

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It was investigated whether treatment with the calcium channels blocker Nimodipine, a 1,4-dihydropyridine derivative, influences vasculopathy and neuronal injury in experimental pneumococcal meningitis. Rats were randomly assigned to one of three experimental groups. The first group (Control group, $n=20$) was given saline (0.4 ml/day) intraperitoneally (ip), whereas the second group (group C, $n=22$) was treated only with ceftriaxone in a dosage of 30 mg/day/ip. The third group (group C-N, $n=22$) was treated with the combination of ceftriaxone 30 mg/day/ip, and nimodipine 0.6 mg/day/ip. Treatment was begun at the time of inoculation in all three groups. The control group was compared clinically and histopathologically with groups C and C-N at 24 h, three and six days after inoculation. Six rats in the control group and eight rats in group C and group C-N were sacrificed at 24 hours and seven rats in each group were sacrificed on the third and sixth day after inoculation. Clinically, there were no significant differences between group C and group C-N ($p>0.05$). There were significant differences between group C and group C-N for vasculopathy and neuronal injury ($p<0.0001$ and $p<0.005$, respectively).

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Significant advances in the antimicrobial therapy of bacterial meningitis have been made in recent years. However, the morbidity and mortality rates attributable to this infectious disease remain high. An area of intense interest is the assessment of new therapeutical modalities that regulate the neuronal and cerebrovascular changes during acute bacterial meningitis [1]. Animal models of bacterial meningitis have increased our knowledge of the complex pathophysiologic mechanisms of the disease [2]. Meningitis can lead to significant and often progressive abnormalities in the host's cerebrovascular system resulting in neurologic impairment or death. Vasospasm of the cerebral arteries was reported in not only tuberculous meningitis but also purulent meningitis patients [3, 4].

Nimodipine, a 1,4-dihydropyridine derivative, is related to nifedipine, but the smooth muscle relaxant effect preferentially acts on cerebral arteries. It inhibits the Ca^{++} influx into nerve and smooth muscle cells via voltage sensitive and receptor channels [5]. It is known that nimodipine has both vasodilator and anti-ischemic effects preventing vascular spasm following subarachnoid hemorrhage [5–7]. In the combined therapy (antibiotic and nimodipine) we expect that nimodipine by means of vasodilatation could result in a higher antibacterial effect on bacterial meningitis. The purpose of the present study, therefore, is to investigate and assess the therapeutic effects of the combined therapy-histologically and clinically on experimental pneumococcal meningitis of rats.

Materials and methods

Model of experimental meningitis. Meningitis was produced by intra-cisternal (ic) injection of 0.3 ml of saline containing an inoculum of 10^6 – 10^7 cfu of *Streptococcus pneumoniae* type 3, as described by Dacey and Sande [8]. The microorganism was isolated from the CSF of an adult patient with acute bacterial meningitis at Dicle University Hospital.

Inoculum preparation. *Streptococcus pneumoniae* was inoculated into 10 ml of brain/heart infusion medium and incubated at 37 °C for 16 h. The cultures were centrifuged at 10,000 rpm in an SS34 rotor for 10 minutes, and the supernatant was discarded. The pellet was resuspended in 10 ml of 0.85% sodium chloride solution and was again centrifuged for 10 min at 10,000 rpm. The supernatant was discarded and the pellet resuspended in 1 ml of the same salt solution. Final concentrations of viable organisms were routinely assayed by serial dilutions with planting on solid media ranging from 2.4 to 5×10^7 colony-forming units (cfu)/ml.

Animals. Experiments were carried out on pathogen free Sprague Dawley rats. The animals were housed and fed under standard conditions. The experimental meningitis was produced by the model described by Dacey and Sande. Briefly, male Sprague-Dawley rats, weighing 250 to 370 gm, were anaesthetized with ketamine (11.6 mg/kg) and xylazine (1.16 mg/kg). Then stereo-tactically inoculated with 0.3 ml of the bacterium suspension in the cisterna magna.

Experimental groups. Animals were randomly assigned to one of three experimental groups. The first group (Control group) was given saline solution (0.4 ml/day) intraperitoneally (ip), whereas the second group (group C) were treated only with ceftriaxone in a dosage of 30 mg/day/ip. The third group (group C-N) were treated with the combination of ceftriaxone 30 mg/day/ip, and nimodipine 0.6 mg/day/ip. Treatment was begun at the time of inoculation in all groups.

Each rat underwent daily neurological examination as well as before, and after inoculation. CSF (5–10 µl) was obtained by puncture of the cisterna magna and the CSF was cultured to document

meningitis. The control group was compared with groups C and C-N at 24 h, three and six days after inoculation clinically and histopathologically. Six rats in the control group and eight rats in group C and group C-N were sacrificed at 24 h and seven rats in each group was sacrificed on the third and sixth day after inoculation. These times correspond to stages of meningitis in humans. Then, the brain was carefully removed. Brains were initially sectioned in a coronal direction. When no inflammation was found at the dural space, sequential sections were made at 1.5 mm distances. Brains without macroscopic pathology were not excluded from the histological study. The brains were fixed by perfusion fixation with phosphate-buffered saline followed by Boun's fixative. The tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Endpoint analyses of clinical and histopathological changes were scored by the method described by Kim et al. [9]. Clinical severity of the disease in every animal after 24 h, three and six days of infection was graded as follows: 0= normal activity/ambulation; 1= reduced ambulation; 2= slow righting; 3= unable to right within 5 seconds. At the same time, the presence or absence of tonic seizures was recorded. Brain sections stained with hematoxylin-eosine were examined for the presence of inflammation in the subarachnoid space and ventricles, and for vasculopathy, defined as vascular engorgement and scored semi-quantitatively by the average number of affected vessels per section (0= none; 1= 1-3 vessels/section; 2= 4-8 vessels/section; 3= >8 vessels/section). Neuronal injury (areas of decreased density of neurons, frank areas of necrosis) was scored semi-quantitatively based on a pre-determined scale (0= 1-2 foci/hemisphere; 2= 3-4 foci/hemisphere; 3= multiple (>4) or confluent foci; 4= >50% of hemisphere affected). Histopathologic evaluations were performed by an investigator blinded to the treatment of animals.

Statistical analysis. Statistical comparisons between the groups were carried out by a Student's *t*-test, and $p < 0.05$ was considered to be significant.

Results

By 24 h after infection meningitis was fully developed in all animals both clinically and microbiologically. All animals were bacteriemic, and all had high CSF bacterial titers. The clinical severity of the disease was assessed in all animals at 24 h, third and sixth day after inoculation. Most of the animals in control group had scores between two and three (Table I). There were significant differences between the control group and the other two groups for clinical course, vasculopathy, neuronal injury and mortality ($p \leq 0.01$ for all groups). There were no significant differences between group C and group C-N for clinical course and mortality. There were significant differences between group C and group C-N for vasculopathy and neuronal injury ($p < 0.0001$ and $p < 0.005$, respectively).

Histopathology

Histopathologically, the disease in the rats in each of the three groups at 24 h after infection was characterized by subarachnoid and ventricular inflammation, vasculopathy, and neuronal injury. A granulocytic infiltration involving the entire subarachnoid and

ventricular space was present in all infected animals without obvious differences between the groups.

On the third day, in the control group and in group C, most of the subarachnoid vessels and their parenchymal branches showed marked dilatation and were filled with erythrocytes (Fig. 1). In group C-N, most of the subarachnoid vessels and their parenchymal branches did not show any dilatation and were not filled with erythrocytes.

In animals treated with ceftriaxone, the level of neuronal injury proved to be more extensive than in animals treated with ceftriaxone and nimodipine (Figs 2, 3). Foci of severe neuronal injury appeared throughout the cortex, commonly in areas showing evidence of vasculopathy.

At the sixth day, the inflammation persisted in the subarachnoid spaces. The most subtle neuronal change at this time point was the ballooning of neurons (Fig. 4). Areas of more severe injured neurons were necrotic and clearly demarcated from areas with preserved neurons. An infiltrate of mononuclear cells were seen in injured areas (Fig. 4). In most of the animals treated with ceftriaxone, the extent of injury varied from small occasional foci to large cortical necrosis involving more than 50% of the cortex and tended to be wedge-shaped.

Table I

Clinical and histopathologic parameters

Parameters	Control group n=20	Group C ¹ n=22	Group C-N ² n=22
Illness score (0-3)			
After 24 hours	2.4±0.7	2.0±0.6	1.9±0.5
After 3 days	2.6±0.5	1.8±0.6	1.7±0.6
After 6 days	2.6±0.5	1.9±0.4	1.7±0.5
Vasculopathy score (0-3)			
After 24 hours	2.7±0.5	2.5±0.8	1.7±0.8
After 3 days	2.7±0.5	1.7±0.8	1.9±0.7
After 6 days	2.6±0.5	2.0±1.0	1.3±0.5
Neuronal injury score (0-4)			
After 24 hours	3.0±0.6	2.2±0.8	1.5±1.6
After 3 days	3.0±0.6	2.4±0.8	1.4±0.5
After 6 days	2.9±0.9	2.3±0.5	1.7±0.5
Mortality No (%)	5 (25.0)	3 (13.6)	2 (9.1)

¹ Ceftriaxone-treated rats² Ceftriaxone + nimodipine-treated rats

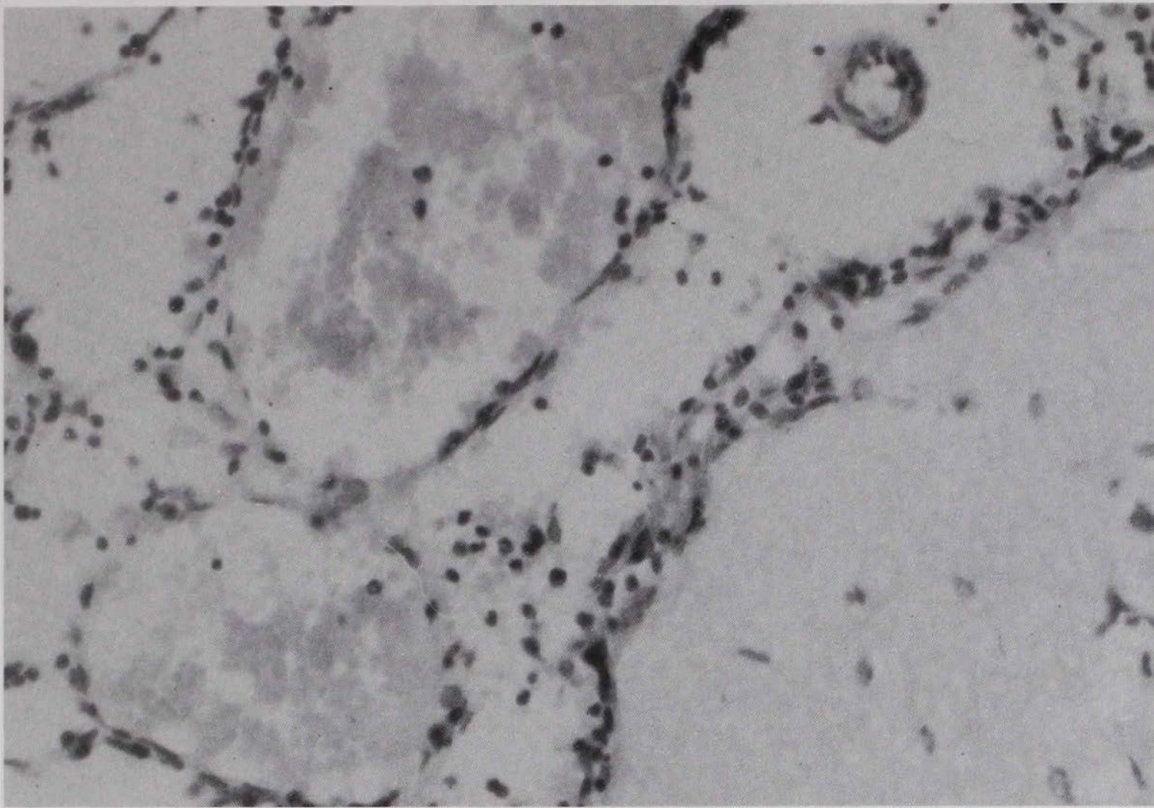


Fig. 1. Vasculopathy defined as vascular engorgement in group C on the third day (Hematoxylin and eosin, $\times 200$)

Discussion

In several experimental meningitis studies, the increase in the permeability of blood-brain barrier (BBB) and the loss of cerebral auto-regulation has been well demonstrated [10–12]. Three phases of cerebral arteriopathy developed in meningitis, resulting in marked histological changes in the arterial wall [13]. Previous morphologic studies have shown an intact arachnoid membrane in animals with experimental meningitis. The increased permeability observed in patients with bacterial meningitis occurs at the level of the choroid plexus epithelium or the cerebral microvascular endothelium, or both [9].

Calcium ions play a major role in the control of micro-vascular tone and in the mediation of the effects of vasoactive agents. Calcium channel blockers are increasingly used in cerebral diseases. Recent attention has focused on Ca^{++} channel blockers such as nimodipine. Following a regional hypoxic insult, therapy should aim at the decrease of local cerebral vasoconstriction and shunt blood to ischemic areas. Nimodipine modulates neuronal activity via the blockade of slowly inactivating (L-type) calcium channels [14].

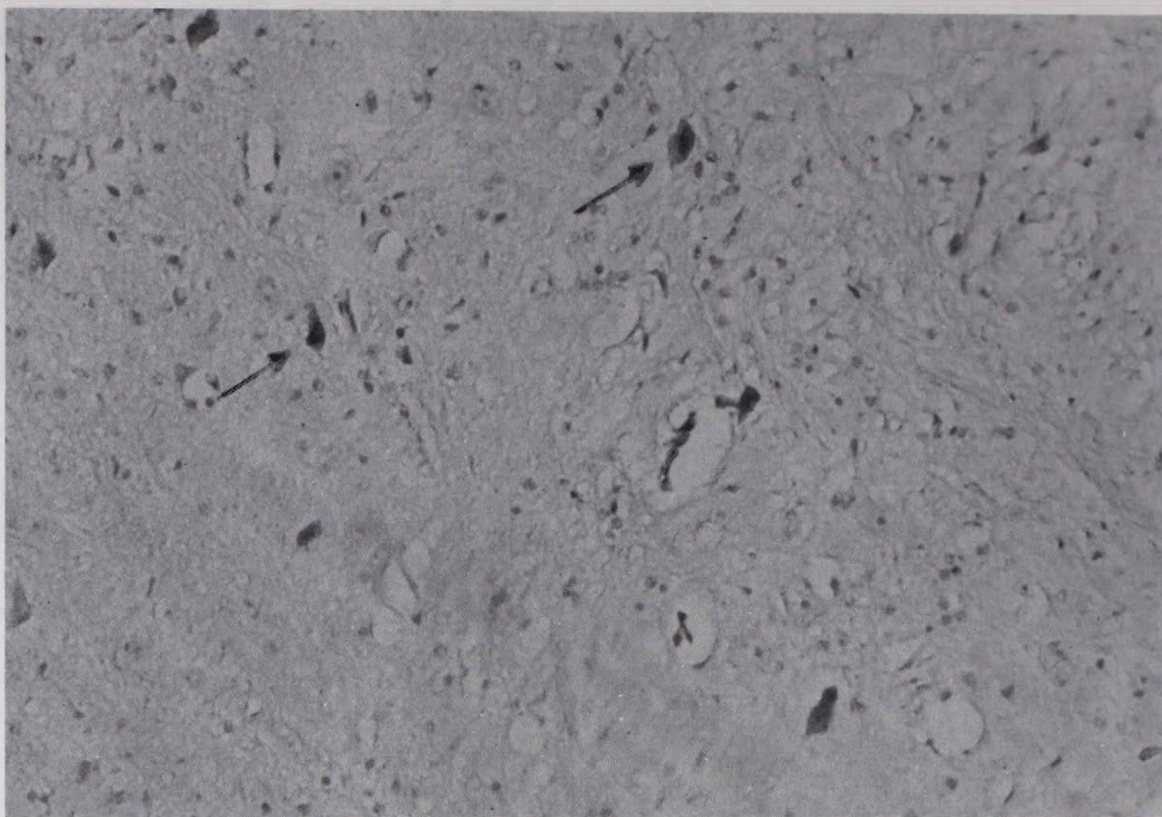


Fig. 2. A few necrotic neurons were seen in group C-N at the third day (Hematoxylin and eosin, $\times 200$)

A significant increase in regional blood flow was found with nimodipine treatment in patients with cerebral vasospasm after subarachnoid hemorrhage (SAH) and nimodipine is increasingly used for the therapy and prophylaxis of cerebral vasospasm after SAH [15]. Blood flow through the cerebral microvessels plays a fundamental role in supplying oxygen and to the cerebral tissue and in the control of cerebrovascular resistance [16, 17]. In criticism, the arterial dilatation was considered to be an attempt of the body to improve collateral circulation to an ischemic area. In the last stage, organic stenosis was seen and considered to be a repair process which was due to the organization of sub-endothelial edema with resultant intimal thickening. In both light and electron microscopic examination, evidences of increased endothelial permeability and necrosis of smooth muscle cells were present [18, 19].

It has been expected that nimodipine could maintain the ceftriaxone flow to the CSF despite reactive vasospasm, because nimodipine has both vasodilator and anti-ischemic effects on cerebral arteries (unpublished data). In our study, vasospasm demonstrated microscopically at 24 h, third and six days after the inoculation is considered to have been evoked by inflammatory exudate.

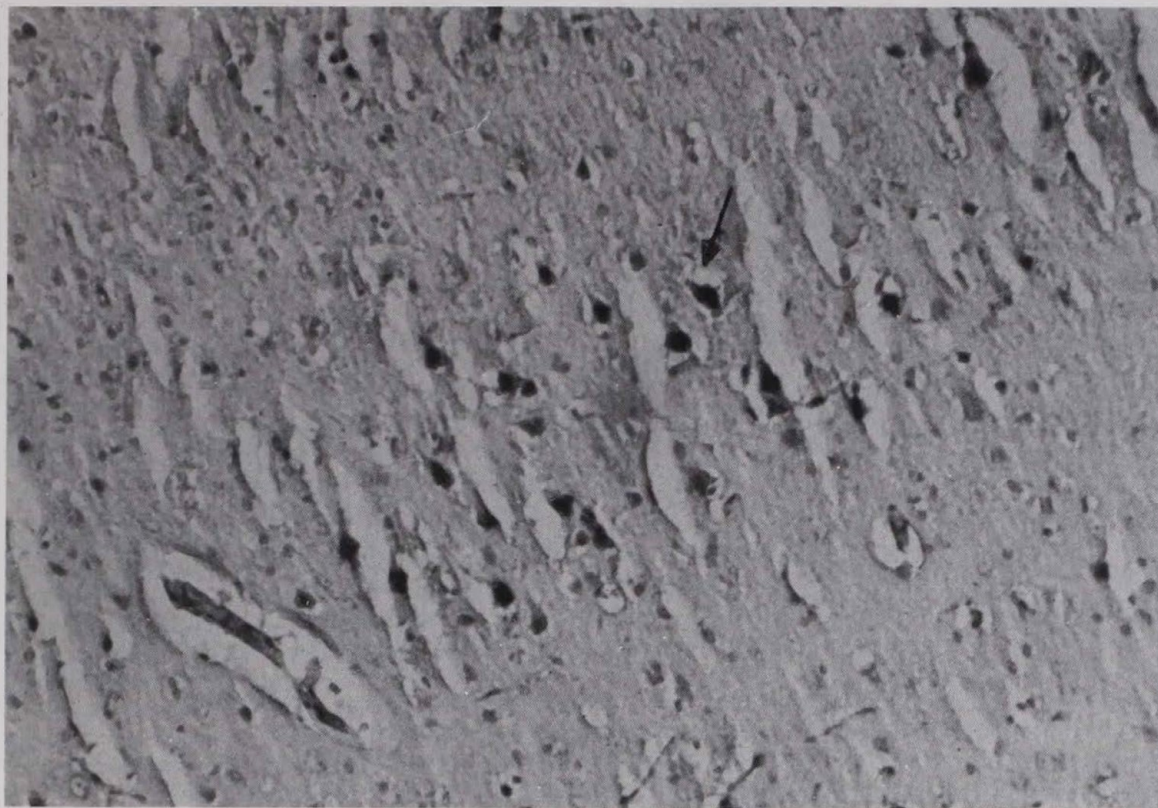


Fig. 3. In group C, more extensive necrotic neurons were shown than in group C-N in Fig. 2, on the third day (Hematoxylin and eosin, $\times 200$)

In this study, not only vasospasm but also vasodilatation was observed in the antibiotic treated group. This condition may be the result of a local loss of autoregulation.

In this study, in spite of the histological differences, there were no clinical differences between the groups which is a contradiction. However, a lower mortality was observed in the group treated with antibiotics and nimodipine than in the group treated with antibiotics only; but this difference was not significant. Similar findings were reported by Kim et al. [9].

In conclusion: Nimodipine has important effects on pneumococcal meningitis, but much more extensive studies are needed before it can be used clinically. Further study is necessary to confirm these preliminary findings and to clearly delineate the mechanisms of action.

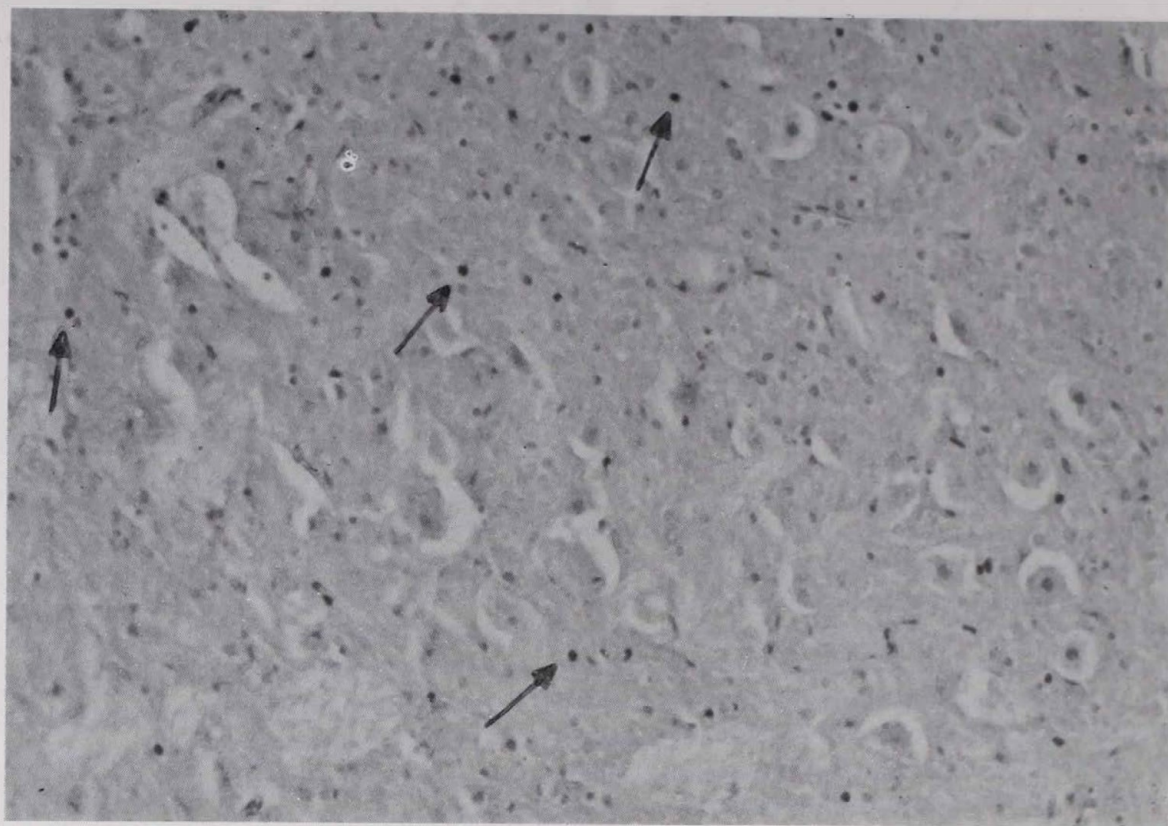


Fig. 4. The ballooning of neurons and mononuclear cells' infiltration on the sixth day (Hematoxylin and eosin, $\times 200$)

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THERMOSTABLE, HIGH SALT-TOLERANT AMYLASE FROM *BACILLUS MEGATERIUM* VUMB-109

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A bacterial strain, *Bacillus megaterium* VUMB-109, has been isolated and identified which produces salt-tolerant, thermostable amylase (16 U/ml culture filtrate). Cultural conditions such as carbon and nitrogen sources, metal ions, temperature and pH have been optimized for enzyme production. The partially purified enzyme was active over a wide range of pH (4.5–10) and exhibited maximum activity at 93 °C, retaining 90% original activity at 100 °C. Partially purified enzyme was stable at 70 °C for 60 min. The enzyme was stable in NaCl up to 5M over 24 h without losing its original activity.

Among the various microbial enzymes used in industrial processes, amylases have wider application and far more importance than any other enzyme. The hydrolytic products of these enzymes are used in the textile, paper, food and pharmaceutical industry. A number of microbial species are potent producers of these enzymes, but only a few of these are thermostable [1–5]. These amylases are active at a wide range of pH values and temperature.

In this paper we deal with a high salt-tolerant as well as thermostable amylase from *Bacillus megaterium* VUMB-109 and some of its properties.

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Materials and methods

Isolation of amylase producing bacterial strain. Soil samples were collected from different Municipal garbage heaps of Midnapore district, West Bengal, India. Several strains of bacteria were isolated on selective medium containing (w/v) 0.2% soluble starch (BDH, India), 0.5% peptone (Difco) 0.3% $(\text{NH}_4)_2\text{HPO}_4$ (BDH, India), 0.1% NaCl and 1.5% Agar (BDH, India). The pH of the medium was adjusted to 8.2. Plates were incubated at 43 °C for 24h. Amylase producing strains of bacteria were detected from the plates by the formation of clear zones around the colonies after flooding the plates with Iodine (1% I_2 w/v in 2% (W/V) KI) solution. *Bacillus megaterium* VUMB-109 was selected as the best amylase producer and was used for further studies. The strain was maintained at 4 °C on agar slants containing the medium used for its isolation.

Preparation of inoculum. A standard inoculum was prepared by growing the organisms in 50 ml medium (see above) in 250 ml Erlenmeyer flasks for 12 h on a rotary shaker (200 rev/min) at 43 °C. Organisms at log phase (OD 2.8 at 620 nm) were used as a source of inoculum.

Production of enzyme. Enzyme production was carried out by growing the bacilli in 250 ml Erlenmeyer flasks containing 50 ml liquid medium (pH-8.2) having identical composition with the isolation medium. A 1% inoculum was added and incubated at 43 °C for 28 h in a rotary shaker at 200 rpm. The culture supernatants obtained by centrifugation were assayed periodically for amylase activity. Enzyme production was tested in the presence of various carbon sources, organic nitrogen sources and metal ions. To determine the optimum temperature for enzyme production, fermentation was carried out at various temperatures (37 °C to 55 °C). To determine the optimum initial pH for enzyme production the medium was adjusted to various pH by the addition of 1(N) HCl or 1(N) NaOH. Growth of the organisms was estimated on the basis of changes of optical density at 620 nm using a Klett Summerson colorimeter along with enzyme production.

Partial purification of amylase. After 28 h cultivation on a rotary shaker (200 rpm) at 43 °C, the fermented broth was centrifuged to obtain the culture supernatant. The supernatant was then treated with solid ammonium sulphate (40–80% saturation) and allowed to stand overnight. The precipitate formed was collected by centrifugation ($15000 \times g$, 15 min 4 °C), dissolved in phosphate buffer (10 mM, pH 7.75) and dialysed against the same buffer for 24 h at 4 °C. The dialysate was used as the source of enzyme.

Assay of amylase. Amylase activity was determined by the method of Bernfeld [6]. The reaction mixture consisted of 0.5 ml of 1% starch, dissolved in 0.4 ml of 10 mM phosphate buffer (pH 7.75) and 0.1 ml of enzyme solution. The reaction mixture was incubated at 93 °C for 5 min and the reaction was terminated by immediate cooling in ice and adding 1 ml of 3,5-dinitrosalicylic acid. Absorbance was measured at 540 nm.

One saccharolytic unit was defined as that amount of enzyme which produces 1 μmol reducing sugar equivalent to glucose per minute under optimal assay condition.

Effect of temperature on amylase activity and stability. To determine optimum temperature, amylase activity was measured at different temperatures for 5 min at pH 7.75. For thermal stability the enzyme solution was held at different temperature for 60 min in phosphate buffer (pH 7.75, 10 mM) and then cooled immediately in ice and the residual activity was measured at optimum temperature.

Effect of pH on amylase activity and stability. To determine pH, amylase activity was measured at different pH values at 93 °C for 5 min. For pH stability the enzyme solutions at a desired pH were kept at 4 °C for 24 h and then measured at optimum temperature.

Salt tolerant test. Enzyme was incubated in 10 mM phosphate buffer (pH 7.75) containing various NaCl concentrations (0 to 5 M) for 24 h at 4 °C and the activities were measured in the same way as mentioned above.

Effect of metal ions on enzyme activity. Various metal ions (at 5mM) were added to the standard assay mixture and activity of the enzyme was measured in the same way as mentioned above.

Results

Identification of organism. The organism (VUMB-109) was identified as *Bacillus megaterium* based on the morphological and physiological characteristics [7]. Identification of the isolate was also confirmed from the Institute of Microbial Technology (MTCC), Chandigarh, India.

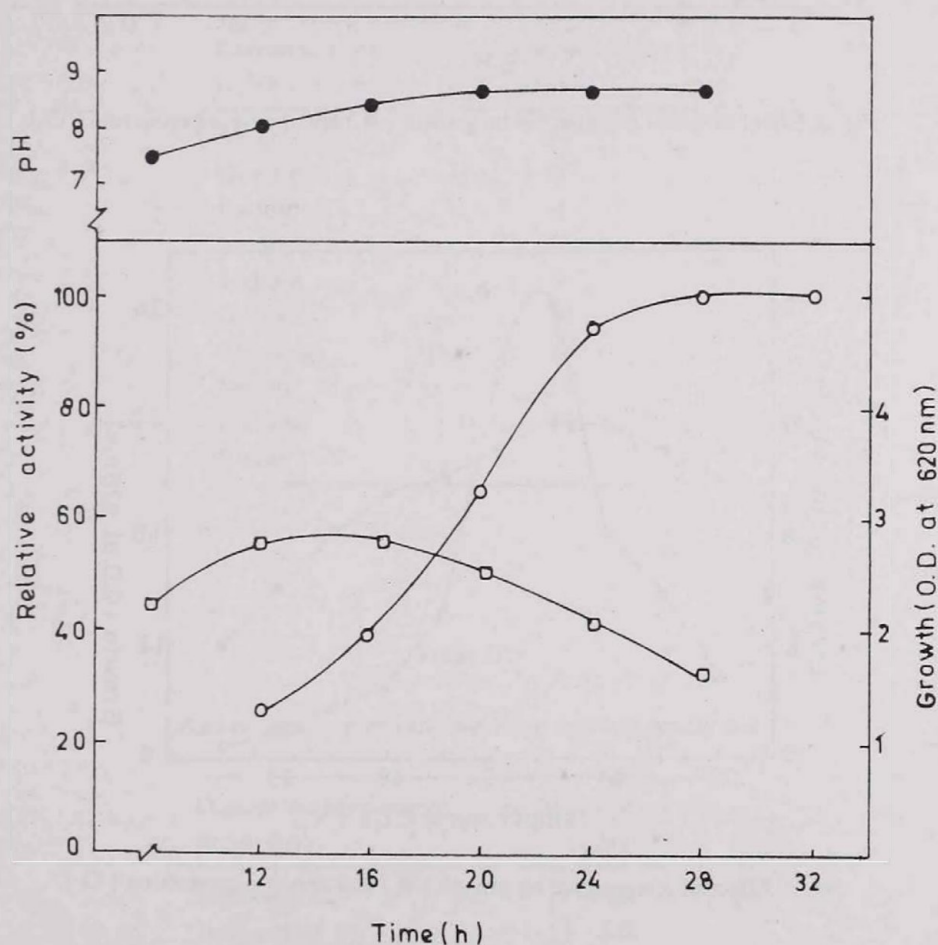


Fig. 1. Time course of cell growth (□) amylase activity (○) and pH (●) by *Bacillus megaterium* VUMB-109

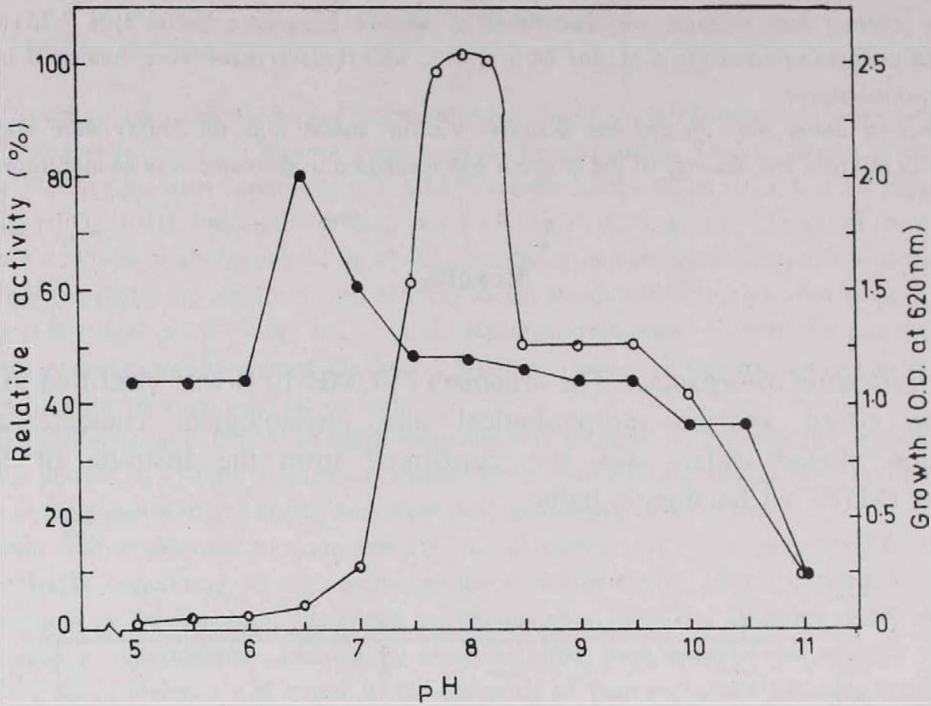


Fig. 2. Effect of initial medium pH on growth (●) and amylase production (○)

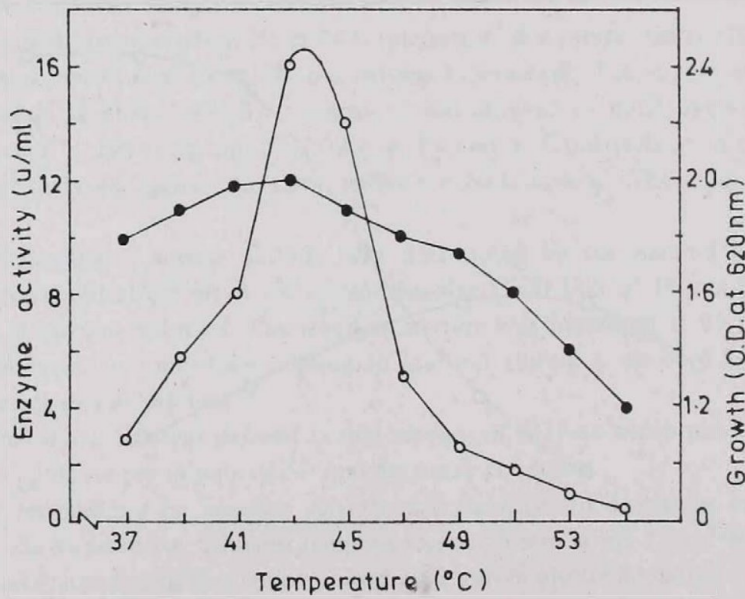


Fig. 3. Effect of temperature on growth (●) and amylase production (○)

Amylase production. Amylase production was started from 10 h of growth and reached a maximum in 28 h (Fig. 1). Though the growth of the organism ceases after 16 h, the enzyme production increases up to 28 h. The pH of the broth increased from 8.2 to 8.5 until the end of the fermentation.

Effect of pH and temperature on enzyme production. Enzyme production was observed between pH 7.0 and 11.0. Maximum enzyme production and growth were observed at initial pH 8.2 and 6.5, respectively (Fig. 2). At higher pH values (9.5–11.0), the growth of the organism as well as the amount of enzyme production was low. The optimum temperature for growth and enzyme production was found to be 43 °C at pH 8.2 (Fig. 3). Growth and enzyme production started to decrease drastically above 45 °C.

Table I

Effect of carbon sources added to basal medium on amylase production

Carbon sources (0.5% w/v)	Saccharolytic activity (U/ml)
Starch	16
Glucose	2
Lactose	4
Xylose	14
Maltose	9
Dextrin	14
Rhamnose	5
Inositol	14
Fructose	14
Ribose	2

Table II

Effect of organic nitrogen sources on amylase production

Organic nitrogen source (0.5% w/v)	Saccharolytic activity (U/ml)
Peptone	13.5
Beef extract	8.0
Yeast extract	3.0
Tryptone	3.0
Meat extract	1.0
Malt extract	8.0

Table III

Effect of metal ions on amylase production

Metal ion concentration (0.1% w/v)	Saccharolytic activity (U/ml)
NaCl	13
KCl	15
MgSO ₄ ·7H ₂ O	16
CaCl ₂	1
FeCl ₃	1
BaCl ₂	18
CuSO ₄ ·5H ₂ O	0
HgCl ₂	0

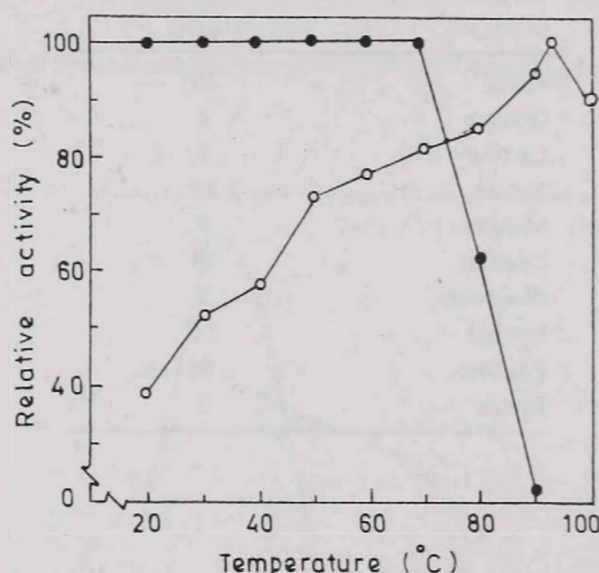


Fig. 4. Effect of temperature on amylase activity (○) and stability (●)

Effect of carbon source. Effect of different carbon sources on enzyme production is shown in Table I. Starch, dextrin, xylose, inositol, fructose proved to be good carbon sources for enzyme production, whereas production was not induced by glucose, maltose, arabinose and ribose. Rhamnose and lactose were poor inducers of enzyme production.

Effect of nitrogen sources. Various nitrogen sources at 0.5% were tested for amylase production (Table II). Among the various organic nitrogen sources, peptone was found to be the best followed by beef extract, malt extract, yeast extract, tryptone and meat extract.

Effect of metal ions. Additive effect of different metal ions (at 0.1% w/v) to the culture medium was tested for enzyme production (Table III). Ba^{2+} , Mg^{2+} , K^+ , Na^+ stimulated enzyme production, whereas Fe^{3+} , Cu^{2+} , Hg^{2+} and Ca^{2+} had no effect.

Properties of the enzyme. The partially purified enzyme was active even at 100 °C, however it showed optimum activity at 93 °C and the enzyme was stable up to 70 °C for 60 min (Fig. 4), but at 80 °C only 62% of the original activity was retained after this time.

Optimum pH for enzyme activity is demonstrated (Fig. 5). The enzyme remained active between pH range 6.0–8.0 but at more acidic or alkaline pH it lose activity very rapidly. The enzyme was stable in NaCl up to 5 M (Fig. 6).

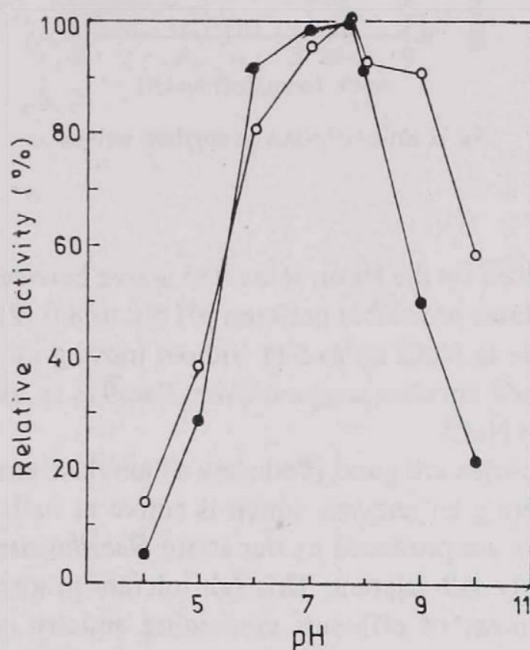


Fig. 5. Effect of pH on amylase activity (○) and stability (●)

Discussion

The strain is a producer of an extracellular starch degrading enzyme. The enzyme is highly thermostable and salt tolerant. Optimization of fermentation conditions were undertaken to increase the production of the enzyme by the strain. From the results it was observed that optimum conditions for the production of this enzyme were at pH 8.2 and at temperature 43 °C whereas the optimal assay conditions were at pH 7.75 and at temperature 93 °C.

In an earlier report an optimum temperature of 91 °C for enzyme activity of *Bacillus licheniformis* was reported [1]. It was also shown that this amylase was stable at

65 °C for 60 min. Our findings are in agreement with the reported one. So far as the thermal inactivation of the enzyme and the effect of temperature on enzyme activity were concerned, the enzyme produced by the strain could be considered as highly thermostable.

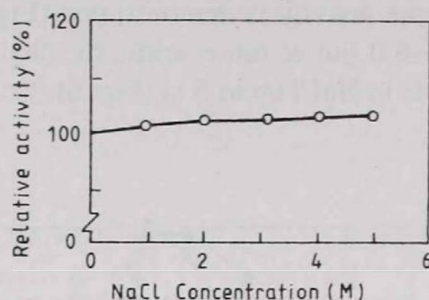


Fig. 6. Effect of NaCl on amylase activity

The enzyme produced by the strain remained active between the pH range 6.0–8.0. Most of the *Bacillus* amylases are stable between pH 5.0 to 8.0 [2]. The enzyme produced by the strain is fairly stable in NaCl up to 5 M without losing its original activity. Earlier Khire and Pant [8] obtained amylase enzyme from *Bacillus* sp. 64 which retains 80% of the original activity in 5 M NaCl.

Several *Bacillus* species are good producers of amylase enzymes but only a few of them are capable of secreting an enzyme which is active at such high temperature [2, 9, 10]. The thermostable amylase produced by our strain *Bacillus megaterium* VUMB-109 is novel in that it is extremely salt-tolerant. This salt tolerant property of our isolate can be very helpful in the treatment of effluents containing starchy or cellulosic residues in pollution control mechanisms using hard or slightly alkaline ground-water.

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THE DEVELOPMENT AND RESULTS OF ORAL MICROBIOLOGY AT THE SEMMELWEIS UNIVERSITY OF MEDICINE*

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The development of oral microbiology at the Semmelweis University of Medicine started around 1980 in the Institute of Microbiology. The teaching of oral microbiology was introduced to the dental educational program, basic research was initiated, appropriate methods learned and used. An intensive collaboration with the Department of Conservative Dentistry developed, which resulted between 1983 and 1995 in about 23 publications (the majority in international journals) and eight lectures, mainly on international forums.

The development and progress of oral microbiology in Hungary was strictly bound to the development of independent dental education. The first dental school, independent from general medical education, was established in 1840, in Baltimore; this was followed by the birth of more, similar dental schools in the USA. By the end of the 19th century independent dental schools formed also in Europe: in Geneva, Paris, in the Scandinavian countries and England. In these countries the oral basic subjects, as oral biology, oral pathology, oral microbiology were created and taught early to dental students, and research work in connection with the specific "oral" subjects began.

In contrast, in several Central- and Southern European countries, dental education was bound to a general medical diploma until the middle of the 20th century*. In Hungary, independent dental education was established between 1955 and 1978 at the four universities, first in Budapest, in the form of the Faculty of Dentistry. Teaching and

* This publication is dedicated to Professors István Nász and Ilona Szeri on the celebration of their 70th birthday. The publication is based on the lecture held at the scientific meeting on May 22, 1997 at the Semmelweis University Medical School.

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research of oral microbiology was initiated first at the Semmelweis University of Medicine, in the second half of the 1970s. The simultaneous development of an oral biology department, the new teaching and research guidelines of Professor István Nász, and the new profile at the Department of Conservative Dentistry were the driving forces for the progress of oral microbiology. By the beginning of the early 80s from the Department of Conservative Dentistry, young dentists (in the course of 10 years subsequently three persons) received the opportunity to learn and practice guidelines and techniques in the Microbiological Institute. By this, a close scientific collaboration between the two Institutes – lasting since 15 years – developed, and is existing still today.

One direction of oral microbiological research focused on the bacterial resistance of dental materials. Orsós et al. [1, 2] reported in 1983 about the results of the resistance of several composite and acrylic based materials against *Pseudomonas aeruginosa*. Of the examined materials, Evicrol, Superlux, and acrylic-based materials proved to be resistant, the filling material Isopast showed a moderate-, Medident teeth and Micromix filling materials medium resistance.

Several light-curing filling materials and glass-ionomer cements were investigated later with similar methods by Herczegh et al. [3]. All the examined filling materials (Visiomolar, Heliomolar, Heliosit, Silux, P-50, Prismatic and Ketac glass-ionomer cements) showed an acceptable resistance against the *P. aeruginosa* strain. After using the oil-test, a slight increase in the colony numbers of the samples – except Ketac silver, which proved to have a bactericidal effect – could be observed.

In another common working group, Csukás, Ferenczi et al. [4] investigated the diffusion of metronidazol used in root canal treatments. Into the root canals of 26 freshly extracted human teeth, 200–400 mg metronidazol has been placed, and the root canal was sealed at both ends. The effect exerted on *Clostridium perfringens* and *Bacteroides fragilis* of the culture medium was examined by the agar-diffusion method. According to the quantitative assessment, the diffusion of the antibacterial agent was continuous, and the concentration of metronidazol in the inhibition zone was about 100 × that of the MIC. The microbiological results supported the good clinical results observed during root canal treatment.

The overwhelming majority of the common research work of the two Institutes was formed by investigations on the microbiology of dental caries, and the microbiological monitoring of preventive programs. These methods were not commonly known in Hungary, therefore Márta Orsós spent several months in the Institute for Preventive Dentistry, University of Würzburg, Ágota Gombik one month at Nijmegen University, and Anna Herczegh five months at the Free University of Amsterdam, in the corresponding microbiological institutes. After these study-trips, and the acclimatization of the corresponding bacterium strains, Orsós et al. reported in 1984 first in this country on the assessments of *Streptococcus mutans* in human saliva [5, 6]. *S. mutans*, *Lactobacillus* and *Candida albicans* were determined from the saliva of 96 adult persons with new, internationally accepted methods, and a positive correlation with the DMF-S mean values, used for the determination of caries prevalence, has been found.

With the determination of microbiological parameters in saliva and dental plaque, the monitoring of caries-preventive programs became possible. Microbiological investigations were used for the first time in Hungary in connection with the xylitol

preventive program, running between 1981–1984, performed in collaboration with the World Health Organization and the University of Turku [7, 8]. Assessing the *S. mutans* counts in the test groups, an increasing tendency of colony numbers with age has been observed. The number of *S. mutans* was significantly less in the xylitol group, when compared with the control.

The in vivo effect of amine-fluoride/stannous-fluoride containing dentifrices has been investigated several times. Bánóczy et al. [9] found no differences in the *S. mutans* counts of saliva after six weeks use of the dentifrices in 25–25 adult persons, between test and control groups. Another study of five months brought similar results [10]. A study performed on a bigger group of younger children, showed however a statistically significant decrease of the bacterial counts after the simultaneous use of the dentifrice and mouth-rinsing (Meridol) [11].

In preventive programs directed to the consumption of fluoridated milk, the children consuming regularly fluoridated milk showed a significant decrease in *S. mutans* counts of dental plaque and saliva compared with the control group [12, 13].

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MALIGNANT LYMPHOMA ARISING FROM NATURAL KILLER CELLS: REPORT OF THE FIRST CASE IN 1970 AND NEWER DEVELOPMENTS IN THE FASL→FASR SYSTEM*

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In 1970 at the University of Texas MD Anderson Hospital, Houston TX, we attended a patient with malignant lymphoma and "lymphosarcoma cell leukaemia". The malignant cells were large with fine granular cytoplasm. The patient was febrile without positive cultures and exhibited wasting resembling "graft-vs-host" disease. Both his lymphoma cells collected from the blood, and his serum, exerted strong cytotoxicity toward a battery of human tumour cell lines established in culture. We reported him as the first case of a new entity: "cytotoxic lymphoma". Later, elsewhere, natural killer (NK) cells were characterized, and cases of malignant lymphomas arising from large granular lymphocytes were described, as those of NK cells. Malignant NK cells constitutively express Fas ligand (L), both membrane-bound (m) and soluble (s). Upon binding FasL Fas receptor (R)-positive host cells succumb to apoptotic death. Patients with FasL-producer lymphoma cells suffer from anaemia, neutropenia, hepatotoxicity and autoimmunity.

The FasL→FasR system regulates normal lymphocyte homeostasis. This system is used by the placental trophoblast against maternal lymphocytes; by transplants against host lymphocytes; and by donor lymphocytes exerting graft-vs-host-reaction. In tumour immunology, FasR⁺ tumour cells can be killed by FasL⁺ lymphocytes and, vice versa, FasL⁺ tumour cells can induce apoptotic death of FasR⁺ host lymphocytes ("counterattack on lymphocytes"). Some tumour cells co-express FasL, FasR and *bcl-2* and not only escape programmed cell death but also utilize the FasL→FasR system as an autocrine growth loop ("counterattack on apoptosis").

* This publication is dedicated to Professors István Nász and Ilona Szeri on the celebration of their 70th birthday. The publication is based on the lecture held at the scientific meeting on May 22, 1997 at the Semmelweis University Medical School.

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We have learned a great deal about the FasL→FasR system as it regulates normal lymphocyte homeostasis from Dr. E. Podack's lectures given in 1994 in Tampa and Orlando FL [1]. For example, CD4 T cells produce IL-2 and thus expand an immunoreactive clone of CD8 T cells. When the immune reaction is accomplished, CD4 cells express FasL and bring the immune reaction to an end by inducing apoptotic death of the FasR⁺ (APO-1; CD95) CD8 cells [2]. However, this question remained unanswered for some 2 years: "what happens when a normal FasL⁺ lymphocyte population undergoes malignant transformation? will it continue to express FasL and thus kill normal (perhaps defensive-reactive) FasR⁺ host lymphocytes"?

During this time immunologically privileged sites (ocular chambers; gonads) were found from where FasL clears FasR⁺ lymphocytes [3]. Transplants were recognized to express FasL and thus protect themselves against rejection by FasR⁺ recipient's lymphocytes [4, 5]. The severe lymphocyte depletion in the host during graft- versus-host disease was found to be mediated by FasL⁺ donor lymphocytes [6, 7]. The trophoblast in the placenta was shown to express FasL, presumably to protect the fetus against rejection by maternal lymphocytes [8]. The first description of an attack mediated by FasL on the host of malignant NK cells appeared early in 1996 [9]. This publication induces us to recall a patients of ours whose case we presented in 1970 as representing a new clinical entity: "cytotoxic lymphoma" [10–12].

Material and Methods, Results

The patient (RS #62557) was a middle aged man, very ill with fevers but with negative blood and urine cultures. He had hepatosplenomegaly and lymphadenopathy. His blood was invaded by large malignant lymphocytes displaying finely granular cytoplasm. He was wasting as if he had graft-versus-host disease (though, he had never received a transplant or a whole blood transfusion). We diagnosed "lymphosarcoma cell leukaemia". The patient's malignant lymphocytes were collected from his blood and were placed on sheets of human tumour cell lines established in permanent cultures [13]; his native (not heat inactivated) serum samples were tested similarly (Experiment 3rd, #2099 12•26•1970).

Slides on which the tumour cells grew in 10% fetal calf serum-containing media were stained daily and the attached and growing tumour cells were counted to construct growth curves for control cultures (no lymphocytes added; or lymphocytes from healthy individuals added) and in experimental cultures (patient RS' lymphocytes added; other patients' lymphocytes added). The initial ratio was 100 lymphocytes per 1 tumour cell; patient RS' lymphocytes were found to grow in these media too; normal lymphocytes persisted and/or showed 1 or 2 generations of growth only. Target tumour cells were as shown in Fig. 1. Both malignant lymphocytes and serum samples of patient RS exerted strong cytotoxicity to most of the target tumour cells, whereas control cultures (without lymphocytes or sera added) grew rapidly [12]. Control lymphocytes often exerted varying, but lesser, degrees of cytotoxicity on these target cells; sera from healthy individuals were not cytotoxic at all [12].

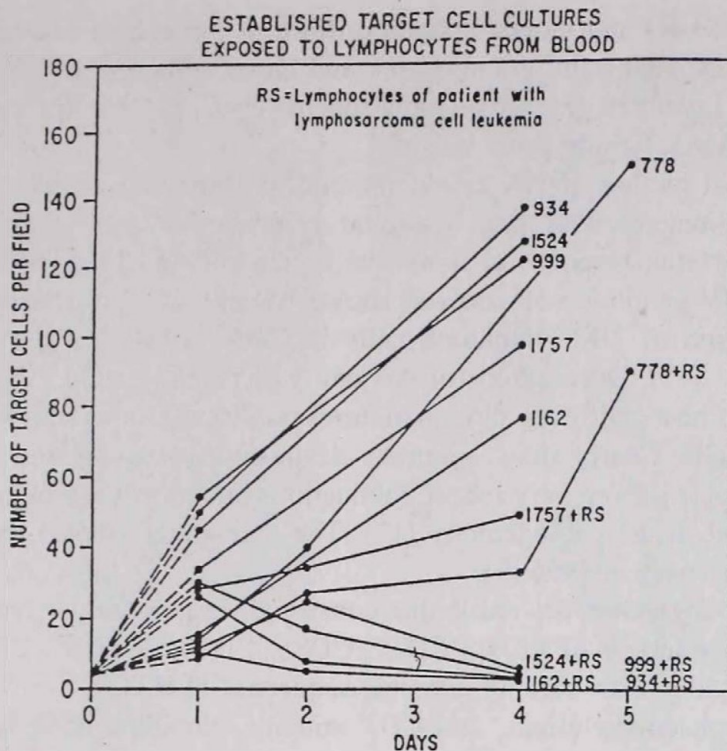


FIG. 3. Growth rate of established cell lines with neoplastic features protocol Nos. 778, 934, 999, 1162, 1524 and 1757 alone and in mixed cultures with peripheral lymphocytes of a patient (Mr. R. S.) with lymphosarcoma cell leukemia. Cell lines 934, 999, 1162 and 1524 are completely, cell lines 778 and 1757 are partially inhibited. Upon prolonged cultivation, cell line 778 appears to gain resistance to the allogeneic cytotoxic lymphocytes.

Interrupted lines: calculated number of target cells in culture. Continuous lines: actual cell counts of target cells on stained and permanently filed coverslip cultures.

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Fig. 1. Original graph and legend showing destruction (and/or growth inhibition) of established human tumour cell lines [13] by malignant lymphoma cells obtained from blood of patient RS. Target cell lines are 778 = malignant lymphoma; 934 = reticulum cell sarcoma; 999 = infectious mononucleosis; 1162 = Henle's fetal human intestine; 1524 = renal cell carcinoma; and 1757 = osteosarcoma.

In other experiments (not shown in this graph) rhabdo- and chondrosarcoma cell lines #1449 and #1459 [13] were also damaged by patient RS' malignant cells. Reproduced from reference [12]

Discussion

Healthy NK cells practice not-MHC-restricted cytotoxicity against their target cells. This effect is primarily mediated by perforins, granzymes and lymphotoxin (TNF β). When the NK cell has accomplished its task, it begins to express FasL and FasR and undergoes apoptotic suicidal death [14]. Malignant NK cells constitutively express

msFasL (but not FasR) and induce apoptotic death of those host cells that are normally FasR⁺ (hepatocytes; neutrophil granulocytes; red blood cells precursors; a subpopulation of lymphocytes). Liver cell damage and neutropenia ensue [9]. Erythrocyte precursors fail to mature. Depletional lymphopenia sets in.

The clinical picture of NK cell lymphoma is characterized by lymphadenopathy and hepatosplenomegaly with large granular lymphocyte count rising up to 50,000. Course is rapidly fatal; response to treatment is insignificant [15]. NK lymphoma cells often harbour EBV genomic sequences as shown by *in situ* hybridization [16]. The most common phenotype of NK lymphoma cells is CD3⁻, CD4⁻, CD8⁻, CD16⁺, CD56⁺, CD57[±], p75IL2R⁺ [15]. Descriptions of this entity as recently as in 1992 fail to mention apoptotic death of host cells even though pictures published, for example liver invaded by NK lymphoma cells, clearly show apoptotic death of hepatocytes and small (reactive?) host lymphocytes [16]. Even very recent publications on this entity fail to elaborate on the expression of FasL in its pathogenicity [17]. The pathogenic role of FasL in this entity was described first early in 1996 [9].

Another malignant entity exists that consists of large granular lymphocytes but of T lineage with phenotype of CD3⁺, CD4⁻, CD8⁺, CD16⁺, CD56⁺, CD57⁻, p75IL2R⁺. These malignant cells often harbour genomic sequences of HTLV 1 or 2. While activated T cells (by phytohaemagglutinin; anti-CD3 immune globulin; IL-2) often temporarily release FasL, malignant T cells of this entity constitutively express the approximately 500 bp genomic sequence that encodes FasL [18]. Clinical presentation of this entity is characterized by neutropenia, autoimmunity with polyclonal hyperglobulinaemia and haemolytic anaemia or red cell aplasia with large granular lymphocyte count seldom exceeding 4000 [15].

Fig. 2. Review of 25 year old scenes of human tumour cell destruction by autologous and allogeneic lymphocytes. Observation: Established cell lines in allogeneic setting are attacked by large granular lymphocytes (now known as NK cells); tumour cells in primary cultures in autologous setting are attacked by smaller lymphocytes without granular cytoplasm (now known as immune T cells) [31, 32]. All preparations shown were stained by Wright:

- A. Embryonal rhabdomyosarcoma cell line (McAllister) #2089 (received from American Type Culture Collection on 12•14•1970) is destroyed by allogeneic lymphocytes of patient RA (MDAH #85779) with rhabdomyosarcoma. Lymphocytes retain good tinctorial characteristics and viability (Experiment #2875 3•8•1972).
- B. Fibrosarcoma cell line T2 (from Dr. Jose Trujillo) carried by us as culture #2117 resists destruction by allogeneic lymphocytes of patients JS (MDAH #82358) with neurofibrosarcoma: nuclei intact; cytoplasmic lysis questionable. Lymphocytes approaching sarcoma cells disintegrate some with nuclear clumping (arrows). (Experiment #2950 4•3•1972)
- C. Primary culture of rhabdomyosarcoma cell of patient MM (MDAH #79161) undergoes some mild vacuolization while autologous lymphocytes show semilunar nuclear clumping and cytoplasmic vacuolization (arrows). (Experiment #2494 10•2•1971)
- D. Primary culture of melanoma cell of patient KC (MDAH #90640) exposed to autologous lymphocytes: target cell dies with nuclear clumping and cytoplasmic vacuolization; some lymphocytes show nuclear clumping and disintegrate (arrows). (Experiment #3024 4•24•1972)

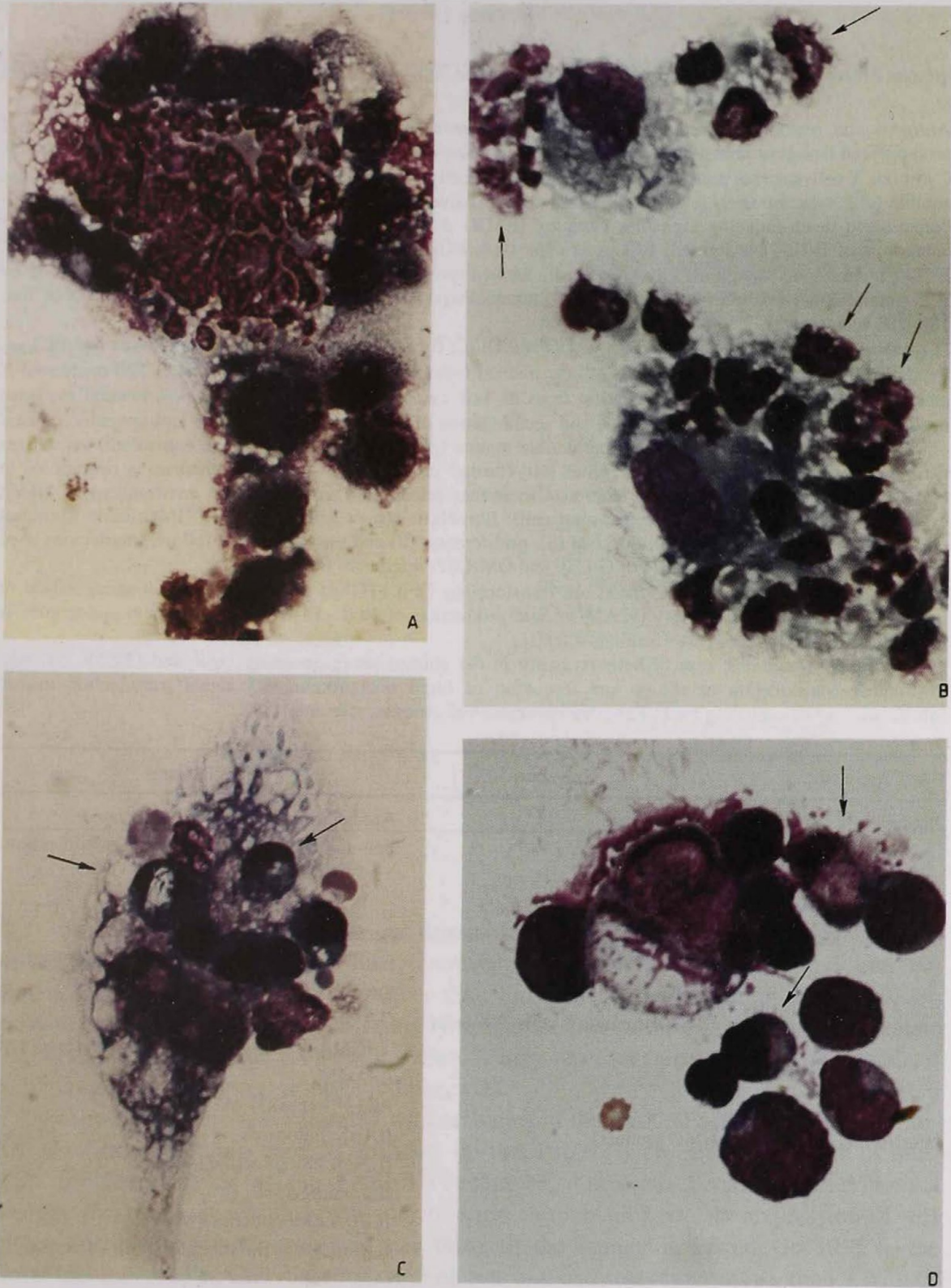
*Fig. 2*

Table I

Human Melanoma Cells (HMC) exerting anti-lymphocyte, anti-apoptotic and proliferative-metastatic activities

Antigens and lymphocyte-mediated cytotoxicity. Human melanoma cell (HMC) expresses ganglioside and proteoglycan antigens that elicit host immune reactions (both antibody- and lymphocyte-mediated) [54]. Cytotoxic T cells express perforins, tumour necrosis factor/lymphotoxin (TNF β) and membrane-bound (m) and soluble (s) Fas ligand (FasL). FasL captured by FasR induces apoptosis (A) as follows: Fas-associated death domain and death-inducing signaling complex (FADD & DISC); BAX/BAX homodimer (death promoter antagonist of BCL-2) is formed; BCL_{XS} is expressed; BCL-2/BCL_{XL}-associated death promoter (BAD) and BCL-2 homologous antagonist killer (BAK) are formed (presumptions) [55]. Endogenous IL-1 β is induced by ICE (interleukin-1 converting enzyme). [56]. Endonucleases are generated to clump and fragment DNA into 180–200 bp integers.

"Counterattack on A" [38]: escape from FADD and DISC; formation of BCL-2/BCL-2 homodimer and BCL_{XL}. Presumed but very likely anti-A events follow: nuclear factor kappa B (NF κ B) antagonist of TNF α -induced A attaches to DNA [57–59] after separation from its I κ B cytoplasmic complex (more experimental evidence needed to show depletion of cytoplasmic and accumulation of intranuclear NF κ B in the anti-apoptotic process of HMC); gene product protein of murine double minute (*mdm*) chromosome (human equivalent) neutralizes gene product protein of p53 as in sarcomas [60] (further proof needed if same mechanism is operational in HMC). In the presence of growth factors (GF) (either released in vivo from the environment of HMC: astrocytes; macrophages; keratinocytes; mast cells; fibroblasts etc. or activated within HMC: basic fibroblast GF) *c-myc* acts as an anti-A agent. Survival (S), proliferation (P) and metastases (M) follow. Interleukins used as autocrine GF: IL-2, 6, 8 [42–45]. For G-CSF and GM-CSF: references [40, 61].

Antilymphocyte actions of HMC listed are transforming GF β (TGF β) production [49]; downregulation of intercellular adhesion molecule-1 (ICAM-1); and production of FasL. FasR⁺ host lymphocyte undergoes A under the effect of FasL of HMC origin [19, 21].

Chromosomal alterations: loss of heterozygosity in the chromosomes encoding FasR and G-CSF [54, 62]. Postulating translocation as shown and formation of fused oncoprotein with signal transduction toward proliferation upon capturing FasL. For other chromosomal changes: reference [54].

HMC Activity	Subjects	Mechanisms
Antigens	Proteoglycans gangliosides MAGE MART melanotransferrin p97; gp95	Antibody- and lymphocyte-mediated immunity (intensified by active immunization)
Defense or death	Immune T cells; NK cells→HMC	Perforin; granzymes; TNF α β ; FasL ^{Ly} →FasR ^{Mel} ; A ^{Mel} Antibody-mediated through FcR ^{NK}
Anti-lymphocyte	HMC→Immune T cells; NK cells	↑ TGF β ; ↓ IL2R ^{Ly} ↓ ICMA-1 ^{Mel} (by IL2): averting attachment of LFA-1 ^{Ly} FasL ^{Mel} →FasR ^{Ly} ; A ^{Ly} [19, 21]
Interleukins	HMC products	IL1 α β : mitogenic IL2→IL2R: autocrine [42, 43] IL4: mitogenic IL6→IL6R: autocrine [63] IL8→IL8R (β thromboglobulin): autocrine [45] IL15→IL2R autocrine [64]

Continued on p. 301.

Table I continued

Growth factor-R	Exogenous GF (from astrocytes, macrophages, keratinocytes, mast cells, fibroblasts, etc)	bFGF; EGF; KGF; IGF; NGF; TNF α β HGF(SCF) $\rightarrow$ <i>ckit</i> R; PDGF; MGSA; neurotrophins
Growth factor: ligand and R	Endogenous (autocrine loops)	bFGF; TGF α ; MGSA; IL2; IL6; IL8; G-CSF; GM-CSF FasL $\rightarrow$ FasR [37, 38]
Neoangiogenesis HMC products	Endothel	VEGF; TGF β ; TNF α ; bFGF; SCF $\rightarrow$ <i>c-kit</i> R [54]
Integrins	Interactions with matrix	Heparanase; collagenase; tenascin; galactin-1; lysosomal, serine and metalloproteinases; laminin α 2 [54]
pro-A events	FADD/DISC	BAX BAX $\rightarrow$ BCL _{XS} $\rightarrow$ BAD BAK ICE $\rightarrow$ IL1 β (endogenous) DNases [56]; GF ⁻ : $\uparrow$ c-myc ^A
anti-A events	BCL2/BAX	BCL2 BCL2 $\rightarrow$ BCL _{XL} ; NF κ B (?); mdm protein + p53 protein (?); GF ⁺ : $\rightarrow$ c-myc ^{anti-A} $\uparrow$ <i>int/hst</i> : bFGF
Chromosomal alterations	Deletions (loss of tumour suppressor genes)	9p21 ⁻ cycline-dependent kinase inhibitor: p16kD 1p36 ⁻ protein kinase gene complex 6q25 ⁻ Mn-superoxide dismutase Y ⁻ [54]
	Amplifications	7p12-13 $\uparrow$ EGFR [54]
	Breaks and re-arrangements	1p32-34: G-CSFR 10q23-26: FasR 11q22-23: tumour suppressor gene [54]
	Hypothetical translocation	t(1;10)(p32-34;q23-26) resulting in fusion oncoprotein FasR+G-CSFR transmitting proliferative signal upon binding FasL (?) [38]

It is entirely possible that various forms of malignant lymphomas express FasL and operational (biologically active) FasL contributes to the clinical picture and immune depletion of malignant lymphomas other than those of NK cells. Pathologists for long recognized "karyorrhexis" in malignant lymphomas: this is an expression of apoptosis that went unrecognized as such for decades. It may represent programmed cell death of both malignant and normal reactive lymphocytes.

The atmosphere was saturated with activities of the FasL $\rightarrow$ FasR system in the mid-90s. (For example more than 50 abstracts on this subject were presented early in mid-1996 in New Orleans at the FASEB (Federation American Societies Experimental Biology meeting)). It was only a matter of short time to hit upon the expression of this system in malignant tumours other than those of the lymphatic system. In 1995-6, the Good Institute studied first placental trophoblasts and choriocarcinoma [8], then – with us melanoma [19]; a group in Ireland colon carcinoma [20]; in Switzerland melanoma and brain tumours [21, 22]; in Germany hepatocellular carcinoma [23]; and a group in the USA various solid tumours (lung, breast, head and neck and kidney carcinomas etc.) [24].

These reports agree that human malignant cells by expressing FasL can destroy FasR⁺ (but not FasR⁻ and apoptosis-resistant) Jurkat T lymphocytes and FasR⁺-defensive host lymphocytes thus imitating the placenta and gaining the same immune privilege the fetus is extended to. However, a recent report preceding those cited above described first destruction of lymphocytes by tumour cells: human breast cancer cells released a soluble factor (FasL? not as yet characterized in 1995-6) that induced apoptotic death of IL-2-activated NK cells and T lymphocytes. This work was presented by Savary and Ju from MD Anderson Hospital Houston TX in San Antonio TX in 1994 [25]. Other than FasL, human breast carcinoma-associated mucin antigen DF3/MUC1 can induce apoptotic death in activated T lymphocytes [26].

Those who observed lymphocyte-mediated cytotoxicity against human tumour cells in the early '70s occasionally recorded tumour cell populations resistant to cytotoxic lymphocytes [27]. However, the USA National Cancer Institute, while approving studies on the mechanism of the phenomenon, failed to release funds for financial support of such projects [28].

Viewing and reinterpreting 25 year old scenes of human tumour cell destruction by lymphocytes from the early '70s [29], we see ghosts of destroyed tumour cells overridden by healthy appearing lymphocytes capable of killing more tumour cells upon a new encounter. We also observe undamaged tumour cells surrounded by lymphocytes that show semilunar nuclear clumps or dissolve in their entirety (Figs 2A-D). Now we know of several antilymphocyte activities that tumour cells may exert. These are (i) oversaturation of T cell receptors by solubilized tumour antigens (a form of "immune paralysis") [30]; (ii) non-expression by tumour cells of MHC molecules and peptide antigens within their grooves; (iii) production of TGF β by tumour cells antagonizing T cell clones by downregulating their IL-2 receptors as these clones expand under the effect of IL-2; (iv) downregulating ICAM-1 expression in the tumour cell: when this happens, the ligand LFA-1 cannot be fitted into the ICAM and the LFA- (CD11a-) expressor lymphocyte lying there idle eventually kills itself by programmed cell death; and (v) the killing of FasR⁺ lymphocytes by FasL-expressing tumour cells [8, 19-24].

One of our projects [33] conceived in 1994 was to introduce by retroviral vector an excessive dosage of the FasR gene into tumour cells and thus render them highly susceptible to the anti-FasR monoclonal antibody that induces apoptotic death upon binding to FasR. However, when human solid tumours were tested, some showed accelerated growth upon encounter with this antibody [34]. Thereafter, neuroblastoma cells were found to utilize TNF as one of their growth factors [35]. This paradoxical situation, i.e. a major inducer of programmed cell death in normal cells now functions as a cell proliferation inducer in malignant cells (neuroblastoma; osteosarcoma and others) [36] forced us to abandon our project. Instead we initiated studies to determine how tumour cells expressing both FasL and FasR escape apoptotic death and if in this setting the TNF α -related FasL functioned as a growth factor. Most recent results indicate that such systems exist in melanoma cells [37, 38]. While important experimental data are still missing, we are able to postulate the chain of events that leads to the survival and proliferation of FasR⁺ melanoma cells upon capturing FasL (Table I) [38]. Furthermore, we suspect the formation of a fusion oncoprotein within melanoma cells. Such fused receptor for Fas and G-CSF could be constructed in vitro [39]. In melanoma cells,

chromosomes encoding for these two receptors suffer loss of heterozygosity and thus translocations as shown in Table I are possible. A melanoma cell line expressing G-CSFR and using G-CSF as one of its growth factors does exist [40]. If such a fusion oncoprotein is formed in melanoma cells it may pose as a "non-self" entity thus inducing immune reactions of the host directed against the cells expressing it (similarly to the BCR/ABL fusion oncoprotein in chronic myelogenous leukaemia) [41].

Apoptosis-resistant lymphomas form BCL-2 homodimers and/or translocate the *bcl-2* gene: t(14;18)(q32;q21). It appears that malignant tumours (here we use as example malignant melanoma) imitate the placenta and expropriate those survival tactics by which lymphocytes pose as memory cells and/or destroy attacker host lymphocytes. Upregulated TNF-Fas systems, certain interleukins [42–45], BCL-2 [46–48], TGF β [49] and downregulated or mutated p53 interact closely [50–53] in human malignant melanoma.

Acknowledgement. We thank Professors Ferenc Rozgonyi and Piroska Anderlik for inviting the publication of this article in the volume dedicated to Professors István Nász and Ilona Szeri on the celebration of their 70th birthday.

Abbreviations: A, apoptosis; ABL, Abelson retroviral oncogene; APO, apoptotic; BAD, BCL2/BCL_{XL}-associated death promoter; BAK, BCL2 homologous antagonist killer; BAX, death promoter antagonist of BCL2; BCL/*bcl*, B cell lymphoma; BCR, chromosomal break point; bFGF, basic fibroblast GF; CD, cluster of differentiation; DISC, death-inducing signaling complex; EBV, Epstein-Barr virus; EGF, epidermal GF; FADD, Fas-associated death domain; Fas, fibroblast and spleen; GF, growth factor; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage (monocyte) stimulating factor; HGF, hepatocyte GF; IGF, insulin-like GF; ICAM, intercellular adhesion molecule; IL, interleukin; int/hst, integrated/human stomach tumour; KGF, keratinocyte GF; L, ligand; LFA, leukocyte function antigen; MAGE, melanoma-associated antigen; MART, melanoma antigen recognized by T cells; mdm, murine double minute (chromosome); MGSA, melanoma growth stimulating activity; MHC, major histocompatibility complex; NF κ B, nuclear factor kappa B; NGF, nerve GF; NK, natural killer; PDGF, platelet-derived GF; R, receptor; SCF, scatter factor; TGF, transforming GF; TNF, tumour necrosis factor; VEGF, vascular endothelial GF

Appendix. The Editors of the Acta in reviewing Dr. Sinkovics' article decided to reproduce his original presentation (reference 11 in the article): "Cytotoxic lymphoma": do Neoplastic Lymphoid Cells Produce Cytotoxins in Vivo?"

FIRST MEETING OF THE EUROPEAN DIVISION OF THE INTERNATIONAL SOCIETY
OF THE HAEMATOLOGY

Milano, 10-12 September 1971

ABSTRACTS 273

"CYTOTOXIC LYMPHOMA": DO NEOPLASTIC LYMPHOID CELLS PRODUCE CYTOTOXINS IN VIVO? Sinkovics J. G. and Shullenberger C. C. The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, Houston, Texas (U.S.A.)

Lymphoid cells growing "autonomously" in established suspension cultures in vitro manufacture molecular mediators (lymphocytic cytotoxins, etc.) of the delayed hypersensitivity reaction. These cytotoxins are produced "autonomously", i.e. without proper stimulus and control, and cause damage to a battery of target cells apparently in a nonspecific fashion (Sinkovics, Dreyer, Shirato, Cabiness, Shullenberger: Texas Rep. Biol. Med., summer, 1971, in print). We attended a patient with lymphocytic lymphoma: the patient developed lymphosarcoma cell leukemia and "wasting syndrome". Neoplastic lymphoid cells obtained from the circulating blood of this patient exhibited strong to moderate cytotoxic effect, within 24 hours of co-cultivation, on a battery of target cell lines deriving from lymphoreticular neoplasia, sarcoma, carcinoma and on normal human embryonic cell lines. The serum of this patient was also cytotoxic to various cultured target cells. Two other patients with lymphosarcoma cell leukemia and "wasting syndrome" yielded sera that were cytotoxic to various cell cultures. These cultures grew well in normal human sera and suffered moderate damage after several days of co-cultivation with normal allogeneic lymphocytes. We propose that neoplastic lymphoid cells may manufacture in vivo molecular mediators (cytotoxins, etc.) of the delayed hypersensitivity reaction in an "autonomous" fashion. The massive release of these mediators may be responsible for some of the clinical features of lymphoma resembling "infection" and "graft versus host reaction". The identification of lymphocytic cytotoxins in certain types of lymphomas requires further biochemical studies. (Supported by the Paul and Harry Barnhart Fund and by institutional research grant IN 43 L-01 from the American Cancer Society).

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EXTENDED-PECTRUM BETA-LACTAMASES: AN ACTUAL PROBLEM OF HOSPITAL MICROBIOLOGY

(A REVIEW)

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Although there is a variety of mechanisms of bacterial resistance to beta-lactam antibiotics, the most important one is production of beta-lactamases inactivating penicillins and cephalosporins. The classification of beta-lactamases is based on biochemical, enzymological (i.e. molecular structure, inhibitory property, substrate-profile, relative rate of hydrolysis) and immunological characters. Extended-spectrum beta-lactamases (ESBLs) can be derived from TEM or SHV enzymes. These enzymes have now been sequenced and it has been found that relatively few point mutations have occurred in the gene of the TEM and SHV type enzymes. These point mutations clustered in five areas of the gene. The amino acid mutations can alter the conformation, the active site and change the hydrance of beta-lactamase-cephalosporin binding capacity. So the enzyme is able to bind and hydrolyse the third generation cephalosporins. Successive mutation interacted radically increasing the binding capacity of enzymes and confer resistance to newer cephalosporins. The use of these drugs provides a strong selective pressure to develop these mutations. Sporadic nosocomial outbreaks due to strains producing an ESBL led to an epidemic problem in some hospitals resulting in a concurrent dissemination of genes, plasmids or strains. Clinical epidemiological importance and role of ESBLs and emergence of multiply resistance of bacteria of nosocomial importance are discussed in this brief.

When penicillins first became available for therapy the bacterial pathogens were exquisitely sensitive to them. Following the early effectiveness of the antibacterial agents more and more bacterial strains have appeared proving to be resistant to the originally

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effective drugs connected with the selective pressure of the antibiotics introduced in the last 50 years.

The beta-lactam antibiotics (penicillins and cephalosporins) act by inhibiting transpeptidases, the enzymes that catalyse the final cross-linking of peptidoglycan [1]. Penicillins are composed of beta-lactam ring, thiazolidine ring and an amino group [1, 2]. The cephalosporins have 6 membered ring adjacent to the beta-lactam ring and are substituted in 2 places on the 5-aminocephalosporamid acid (ACA) nucleus [2, 3]. The modification of the side-chain, attached to the 7 carbon of the 7-ACA nucleus causes a still wider spectrum of activity and a high stability (for example: cefotaxime, ceftazidime, ceftriaxone contain a 2-aminothiazole group on 7-carbon of the 7-ACA nucleus and an O-substituted oxyimino group on the 7-beta-acyl-side chain) [1, 4].

Three different mechanisms are responsible for resistance to beta-lactam antibiotics. These are the alteration of the penicillin-binding-protein (PBP), the target of the drug situated on the outer surface of the inner membrane of the cell envelope (i), change of membrane permeability resulting a reduced accumulation of the drug (ii), and production of an enzyme inactivating the drug molecule by hydrolyzing the beta-lactam ring and by splitting the aminobond of the ring, before the antibiotics caught the bacterial cell (iii). There are numerous beta-lactamases of different species varying in substrate specificity [1, 5–7].

Beta-lactamases produced by Gram-positive bacteria are extracellular and those of Gram-negative bacteria are located in the periplasmic space [5–7].

Classification of beta-lactamases. First, in 1968, Sawai described penicillinases and cephalosporinases [8]. In 1973, Richmond and Sykes divided the enzymes into five major groups on the basis of substrate profile [9]. In 1976, Sykes and Matthew emphasized the plasmid-mediated beta-lactamases [10]. In 1989, Bush tried to correlate substrate specificity and inhibitory properties with molecular structure [11, 12].

Four groups based on molecular structure are distinguished [11–14]. Beta-lactamases class A have a molecular weight about 30 kD, having serine at their active site. These enzymes are mostly penicillinases. They are inhibited by beta-lactamase inhibitors as clavulanic acid, sulbactam or tazobactam. Their tertiary structures show similarities to PBPs from which they might have evolved. Class B beta-lactamases are metalloenzymes, having zinc-binding thiol-group at their active-site, and are inhibited by EDTA. Beta-lactamases belonging to class C are large proteins of molecular mass about 40 kD, and they have serine at their active site, too. They are mainly active against cephalosporins. Both class A and C enzymes developed from PBPs but they have slight similarity to each other. Enzymes of class D are oxacillinases [7, 13].

Bush–Jacoby–Mederios' classification is an updated version, which is based on the currently available beta-lactamase sequences [13, 14]. This classification summarizes the molecular classes, the substrate and inhibitory specificities of beta-lactamases (Table I).

Extended-spectrum beta-lactamases. One of the most important mechanisms of resistance is realized by extended-spectrum beta-lactamase (ESBL) produced by the Gram-negative bacteria. Production of an ESBL serves as the basis of resistance against 7-oxyimino cephalosporins [50, 51].

Table I

Molecular classes, substrate and inhibitory specificities of beta-lactamases

Bush-Jacoby-Mederios group	Molecular class	Preferred substrates	Inhibition by		Genetic localisation	Representative enzymes	References
			Ca	EDTA			
1	C	Cephaloridin	—	—	K, P	AmpC enzymes in Gram-negative bacteria	(15, 16, 17, 18, 19, 20)
2a	A	Penicillin	+	+	K, P	Gram-positive bacteria	
2b	A	Penicillin, Cephaloridin	+	—	P, K	TEM-1, TEM-2, SHV-1, HMS-1, LXA-1, TLE-1, TLE-2, LCR-1, NPS-1, ROB-1, OHIO-1	(21, 22, 23, 24, 25, 26)
2be	A	Penicillin, Narrow-spectrum and extended spectrum cephalosporin, monobactam	+	—	P, K	TEM-3 to TEM-26 SHV-2 to SHV-6, K1 in <i>Klebsiella oxytoca</i>	(27, 28, 30, 31, 32, 33, 34, 35)
2br	A	Penicillin	+,—	+	P	TEM-30 to TEM-36	(36, 37)
2c	A	Penicillin Carbanecillin	+	—	P, K	PSE-1 to PSE-4	(38, 39, 40, 41)
2d	D	Penicillin Cloxacillin	+,—	—	P, K	OXA-1 to OXA-11	(42, 43)
2e	A	Cephalosporin	+	—	K, P		
2f	A	Penicillin Cephalosporins, carbapenem	+	—	K	NMC-A, Sme-1, Imi-1	(44, 45)
3	B	Most beta-lactam	—	+	K, P	L1/Xmaltophilia	(46, 47)
4	?	Penicillin	—	?	K, P	Penicillinase/ <i>P. cepacia</i>	(48, 49)

Among the enzymes having extended-spectrum activity TEM and SHV derivative enzymes are the most widespread. The TEM and SHV enzymes are responsible for the resistance to penicillin and cephalosporin derivatives and monobactams but not to cephamycins and carbapenems [6, 50, 52].

Recently, new ESBLs have been reported. Many of them result resistance to all cephalosporins including cephamycins [14]. Bacteria producing ESBLs have frequently caused nosocomial outbreaks over the world [53–55].

TEM and SHV type enzymes. Extended-spectrum beta-lactamases (TEM and SHV derivatives) can be inhibited by the known beta-lactamase inhibitors [4, 23, 50]. Clavulanic acid is the most effective of the three inhibitors, and resistance to it has never been reported [51, 52]. As observed in the double mutated derivatives of TEM-1 enzyme, the effect of mutations responsible for resistance inhibition is suppressed by those responsible for an extended spectrum. Their difference from parent enzymes lies in having not more than four amino acid substitutions distant from TEM-1, TEM-2, SHV-1 enzymes. Silent mutations may also evolve, whose importance is manifested in course of later mutations [4].

Biological parameters are not altered by conservative mutations or mutation remote from the active site of beta-lactamases. Mutation could be hardly identified unless the resistance profile has been altered. It is assumed that a number of silent mutations may occur frequently. Bacteria would undergo a more radical change in the enzyme, if challenged with 7-oxyimino cephalosporins [50].

The detailed class of ESBL enzymes is based on their biochemical properties and hydrolytic activity. In the course of their mutations three remarkable changes may develop in their function. Four groups of enzymes can be distinguished hydrolysing either ceftazidime or cefotaxime [4, 56].

The enzymes indistinguishable from TEM-1 comprise group "0". It is often observed that these enzymes have a greater ability to hydrolyse cefotaxime than ceftazidime *in vitro*, however the rapid penetration of cefotaxime across the outer membrane compensates for the slower hydrolysis.

The activities of enzymes assigned to group "1" is less degree and these enzymes have ability of hydrolysing ceftazidime to a greater extent than cefotaxime. The activities of enzymes assigned to groups "2" and "3" are more intensive, the difference between groups "2" and "3" manifests itself in their affinities with cefotaxime and ceftazidime. Enzymes of group 2 hydrolyse ceftazidime to a greater extent than cefotaxime. In contrast to group 2, the enzymes of group 3 have a greater hydrolysing effect on cefotaxime than ceftazidime [4].

The spatial effect of point mutation. Numerous ESBL enzymes have been sequenced. Relatively few point mutations were found in the genes of the TEM and SHV-types enzymes responsible for the extended substrate specificity. The site of amino acid variation is placed adjacent to the proposed position of the respective cephalosporin 7-substituent in the course of analysis of structure, provided the beta-lactam molecule is bound at the active site. The shape of the active site is altered by the amino acid mutations directly or indirectly [57–59].

The three dimensional structures of ESBLs have been described [56]. The special structure of the enzyme is defined by eleven α -helix, and five β -strands. In development of active site α -3, α -7, α -9 helices and β -3, β -5 strands take part. The mutations occurring in the genes of different ESBLs change the amino acid sequence and the substrate profile. These point mutations are clustered in five areas of the enzyme located next to the active site: area 1 at the position 104 (adjacent to the α -3 helix), area 2 at the position 164 (adjacent to the α -7 helix), area 3 at the position 205 (on the α -9 helix), area 4 at the position 237–240 (on the β -3 strand) and area 5 at the position 265 (on the β -5 strand), respectively [4, 56, 57].

Substitution of glutamic acid to lysine in area 1 at position 104 has a minimal effect on substrate specificity, if it is present as an only change [60]. A simultaneous change of serine to arginine in area 2 at position 164 results in an increase of ceftazidime MIC (for example TEM-26). Among the arginine at position 164, aspartic acid at position 171 and glutaminic acid at position 171 served a salt-bridge, part of which is made up by the short α -7 helix. Through the loss of arginine, the salt-bridges are disrupted, and so the active site cavity becomes bigger and less rigid. In this way it will be capable of a better binding of 7-oxyimino cephalosporins [57, 61–63].

Changes at position 164 alone can change the MIC, but if there is a change also at position 104 a further increase in MIC of ceftazidime may appear [56, 62].

Positions 238, 239, 240 are found at the end of β -3 strand. Mutations at these positions enlarge the active site, and the binding can evolve with 7-oxyimino cephalosporins. The mutations at positions 237 or 238 cause resistance to cefotaxime in first place, whereas the mutation at position 240 cause resistance to ceftazidime [61].

Change methionine to threonine in area 5 at position 265 is ineffective to MIC of either ceftazidime or cefotaxime. Changes in other areas too may generate an increase in cefotaxime MIC [61].

TEM-derived enzymes. The TEM-derivatives could be divided into two groups, as they are derived from TEM-1 or TEM-2 enzymes. Only one difference was detected between the two enzymes: at position 39 lysine is found in TEM-1 and glutamic acid in TEM-2. Examining different derivatives a serial of resistance patterns have been found but no mutations were demonstrated in more than four amino acids. ESBL activity is an attribute of only TEM derivatives but not of TEM-1, TEM-2 enzymes [62].

As a first step of mutation, TEM-12 differs from TEM-1 [27] by changing arginine to serine at position 164. The newly developed TEM-12 already has low ESBL activity, and a higher MIC of ceftazidime than that of cefotaxime. This mutation evolves through ceftazidime therapy [4].

The mutation of TEM-17, substituting glutamic acid with lysine at position 104 is a potential way to TEM-12. Because of this change MIC of cefotaxime becomes eight-fold higher than that of cefotaxime. This mutation would be of negligible clinical significance [27, 29].

TEM-10 and TEM-26 may evolve from TEM-12 depending on whether glutamic acid changes to lysine either at position 104 or at 204. Both enzymes show identical hydrolytic capacity, having high ESBL activity, and hydrolysing ceftazidime to a higher

degree than cefotaxime. However, TEM-26 has got a higher ceftazidimase activity, because of changing glutamic acid to lysine at position 104 [60, 64, 65].

TEM-6 evolves from TEM-17 by substitution from arginine to histidine. TEM-6 has high ESBL activity, consequently MIC of ceftazidime increased [4]. Mutation of TEM-17 creates TEM-15 and TEM-26 also, by changing in the first case glutamic acid to serine at position 238, and in the second case arginine to serine at position 164. Both enzymes attribute ESBL activity, but TEM-26 has a higher affinity to ceftazime than that to cefotaxime, while TEM-15 inactivates cefotaxime in a higher degree than ceftazidime [4, 35].

TEM-9 evolves from TEM-26, substituting threonine with methionine at position 265. This alteration increases the MICs of ceftazidime and cefotaxime, but MIC of cefotaxime increases to a greater extent [4, 35, 64].

Mutation of TEM-10 at position 237 changes alanine to threonine to create TEM-5. As a result of mutation the affinity of the enzyme to cefotaxime becomes higher [4, 66]. Mutation of TEM-1 at position 238 to change the amino acid from glycine to serine creates TEM-19, an enzyme which has got high ESBL activity, with higher MIC of cefotaxime than that of ceftazidime [4].

Further substitution of glutamic acid for lysine at position 104 creates TEM-15, and TEM-4 evolves from TEM-15 by changing threonine to methionine at position 265. TEM-19, TEM-15 and TEM-4 do not differ in their substrate spectrum [4, 67]. TEM-17 is assigned to group 0. TEM-12 is assigned to group 1. TEM-10 and TEM-26 are classified in group 2. TEM-4, TEM-5, TEM-9, TEM-15 and TEM-19 are classified in group 3 [56].

Accordingly, enzymes evolve in the same way from TEM-2 as from TEM-1. Similarly TEM-7, TEM-11, TEM-13 and TEM-18 developed from TEM-2. Substitution of threonine at position 265 by methionine in TEM-2 creates a TEM-13 enzyme. This mutation is effectively silent, i.e. it has no effect on MIC values [4, 68].

TEM-7, TEM-11 may evolve from TEM-2 depending on whether the amino acid at position 164 changes from arginine to serine (TEM-7) or to histidine (TEM-11). The features of TEM-7 and TEM-12 are identical, and assigned to the group 1, they confer very low ESBL activity. Changing arginine at position 164, the structure of active site can alter, those enzymes bind 7-oxyimino cephalosporins better [28, 58].

TEM-18 evolves from TEM-2 changing glutamic acid to lysine at position 104. These enzymes are belonging to group 0. This change increases the MIC of cefotaxime, but the MIC of ceftazidime is unchanged. This enzyme has no clinical importance [4].

Mutation of TEM-7 at position 104 by changing glutamic acid to lysine creates TEM-X which may evolve also from TEM-18 at position 164 by changing arginine to serine. This mutation can increase the hydrolytic activity. TEM-X would be a logical link to TEM-8 [4].

TEM-Y and TEM-8 possibly originated from TEM-X by changing glutamic acid to lysine at position 240 or glycine to serine at position 238, respectively. The substitution at position 240 does not increase the MIC of cefotaxime, in contrast to the mutation at position 238 which increases the resistance to cefotaxime to a greater extent. TEM-8 may also evolve from TEM-3 by changing arginine to serine at position 164. TEM-X and TEM-Y have not yet been described in clinical isolates [4].

Mutation of TEM-Y at position 237 by changing alanine to threonine creates TEM-24. As a result of this mutation the MIC of cefotaxime becomes higher. TEM-24 has a high ESBL activity, and the MIC of cefotaxime is greater than that of ceftazidime. TEM-8 has similar feature as TEM-24. Both enzymes are assigned to group 3 [4].

TEM-3 is one of the derivatives from TEM-18, it is similar to TEM-8 and TEM-24. TEM-X, TEM-3 and TEM-16 may evolve from TEM-18 depending on, whether arginine changes to serine at position 164 or glutamic acid to serine at position 238 or arginine to histidine at position 164, respectively [4].

Mutation of TEM-3 at position 265 by changing threonine to methionine creates TEM-14. Both enzymes have high ESBL activity [4]. TEM-13 and TEM-18 are assigned to group 0. TEM-7 and TEM-11 are assigned to group 1. TEM-X, TEM-Y and TEM-16 are classified to group 2. TEM-3, TEM-4, TEM-14 and TEM-24 are classified to group 3 [4].

SHV-derived enzymes. The SHV-derived enzymes seem to arise from SHV-1 beta-lactamase by a mutation that alter the configuration of the active site to expand their spectrum of activity [21]. Mutation of SHV-1 at position 238 results SHV-2 [4, 30, 31]. Change of glycine-238 to serine has evolved most likely by therapeutical use of antibiotics, as cefotaxime. In all SHV-derived enzymes, the same amino acid serine has been found at position 238, thus they are certainly derived from SHV-2 [56].

SHV-5 is derived from SHV-2 substituting the glutamic acid to lysine at position 240. This change increases the hydrolytic activity. SHV-5 has a high ESBL activity. The MIC of ceftazidime increases in a greater degree than that of cefotaxime [32, 33].

SHV-3 might be developed either from SHV-2 changing arginine to leucine at position 205 or from SHV-4 changing lysine at position 240 to glutamic acid. The change from arginine to leucine at position 205 has no influence on MIC either of cefotaxime or of ceftazidime [70, 71].

SHV-4 may originate from SHV-5 by the change of arginine to leucine at position 205. This mutation, with the simultaneous changing of glutamic acid to lysine at position 240 can increase the MIC of cefotaxime and ceftazidime [72]. So SHV-4, as SHV-5, has high ESBL activity [4].

The most important mutation within SHV-family is the change of glutamic acid at position 240 to lysine. Only those enzymes, which have lysine at this position, have got ESBL activity. This mutation together with a simultaneous change of arginine to leucine at position 205 can further increase the MIC of cefotaxime and ceftazidime. These two mutations are more likely to be selected by continuing challenge of an SHV-2 producing strain with 7-oxymono cephalosporins, especially ceftazidime [6].

SHV-2 and SHV-3 are assigned to group 1. SHV-4 is assigned to group 2. SHV-5 is classified to group 3 [56].

Genetic background. Genes encoding for beta-lactamase production are located either in the bacterial chromosome or on transposable genetic elements: plasmids or transposons. The genes encoding beta-lactamases are usually located on large plasmids. For example: the genes of TEM-3, TEM-5, TEM-12, TEM-24, SHV-4 and SHV-5 enzymes are located on plasmids, while other genes – TEM-3 and TEM 12 – on

transposons [4, 70, 74, 77]. Other genetic events may also contribute to the resistance mediated by ESBLs. IS1-like elements, that insert into the promoter of the gene for TEM-6 and increase its strength have been found in many strains producing TEM-6 and other TEM-variants, confer resistance to ceftazidime and aztreonam [78].

The plasmid-mediated resistance plays an important role in spread of multiple resistance of pathogenic microorganisms. Rapid increase in the spread of antibiotic resistance within and between species often correlate with conjugative dissemination of specific resistance plasmids [56, 59, 73]. Genes of most prevalent and successful beta-lactamases are often carried by plasmids, which can carry resistance to other antimicrobial drugs, such as aminoglycoside, trimethoprim, sulphonamides, tetracyclines and chloramphenicol, respectively. Because of their relative stability and the ease with their disseminated, plasmid encoding ESBLs might be more common in a nosocomial environment [59, 74–76].

Epidemiology. The 7-oxyimino cephalosporins were introduced into clinical use due to the success of the pharmaceutical industry in developing new beta-lactams resistant to hydrolysis by the known plasmid-determined beta-lactamases in around 1978 in Europe [79]. In 1983, a new enzyme SHV-2 developed from SHV-1 due to cefotaxime treatment was found in Germany in *Klebsiella pneumoniae* and *Serratia marcescens* isolates [80]. One year later CTX-1, hydrolysing cefotaxime, was isolated in France [81]. This was found to be identical with TEM-3. Recently, more than 30 ESBLs have been described during the last few years [74].

Strains producing ESBLs can be observed in numerous species of *Enterobacteriaceae*, mainly in *K. pneumoniae*, but also in *Escherichia coli*, *Salmonella* spp., *Enterobacter* spp., *Citrobacter* spp., *S. marcescens* and *Proteus mirabilis* [75, 76, 82, 83].

As genes determining ESBL are located on transferable plasmids or transposons, the beta-lactamases may be expressed in other bacteria later. Apart from this, differences in the distribution of these enzymes can be seen in different countries all over the world. Several enzymes are widespread in geographically different countries (SHV-2, SHV-4, SHV-5, TEM-6), while others are present only in one or two countries (TEM-10, TEM-12 in the USA, England, TEM-3 in France) [84–86].

In France, *K. pneumoniae* strains producing TEM-3, SHV-4 were responsible for hospital outbreaks. Recently, TEM-24 produced by several strains of *Enterobacteriaceae* have been observed. Three ESBLs, namely TEM-3, SHV-4 and TEM-24 are widely disseminated. In the USA, ceftazidime resistant *K. pneumoniae* producing TEM-10, TEM-12, TEM-26 occurred commonly in outbreaks [82, 83]. In the UK, TEM-12 and TEM-26 enzymes were described. In Germany, SHV-5 enzyme was found produced by *K. pneumoniae* strains. These enzymes were isolated from strains of patients hospitalized in different wards of a single hospital [86]. In France, Portugal and England, 14–16% of *K. pneumoniae*, but less than 0.1% of *E. coli* and other *Enterobacteriaceae* strains produce ESBL [82]. Neither difference in the expression of beta-lactamase nor that in frequency of beta-lactamase mutation can be explained as a raised incidence of ESBL in *K. pneumoniae*. ESBL producer strains have been relatively rarely found in *P. mirabilis*, probably in connection with the low frequency of plasmid conjugation [83].

Most ESBL-containing isolates have come from nosocomial strains, with a prevalence as high as 46% of nosocomial *Klebsiella* isolates in some French hospitals. The emergence of the first ESBL producing *K. pneumoniae* was related to selective pressure exerted by the extensive use of cefotaxime [74]. An outbreak caused by *K. pneumoniae* was reported in USA. Appearance of these endemic strains were in connection with an increased use of ceftazidime [88].

Differences in geographical distribution of ESBL enzymes may be based on different therapeutical and prophylactic protocols. Spread of these enzymes depends on the pathogenicity or virulence of the host strain, the stability of plasmid encoding for their production and the selective antibiotic pressure. Sporadic nosocomial outbreaks caused by ESBL producing strains have been endemic but still not pandemic [84, 87]. Concurrent dissemination of plasmids and genes are a result of sporadic, nosocomial outbreaks [54]. On the one hand, these strains can be formed in hospitals due to the selective pressure of third-generation cephalosporin therapy. Because of plasmids determining multiple drug resistance, the appearance and distribution of ESBLs are not only caused by the selective pressure of the third-generation cephalosporins but also by that of other drugs as other beta-lactams, aminoglycosides or co-trimexazole [88]. On the other hand, they are brought from other long-term care institutions. Colonization of the gastrointestinal or respiratory tracts are enhanced by the selective pressure from widespread hospital use of the beta-lactam antibiotics, and it is followed by an infection. The presence of urinary or arterial catheters, the surgery, the prolonged hospital stay especially in intensive care units can contribute to nosocomial outbreaks. A higher frequency of incidence has been described at intensive care units and oncology units [55, 67].

Other extended-spectrum beta-lactamases. More beta-lactamases having extended spectrum activity were described not derived from TEM and SHV enzymes. These enzymes are rare. Some of them were described only at isolated cases [89].

Non-TEM or SHV derivatives. These new plasmid-mediated ESBLs, belonging to the class A, are PER-1 [90, 91], CTX-M1, CTX-M2 [26, 92] and MEN-1 [20, 44] enzymes. They are very similar to each other. MEN-1 has a 72% homology with chromosomally encoded enzymes of *Klebsiella oxytoca*. CTX-M1 and CTX-M2 differs only by their pI and their susceptibility to beta-lactamase inhibitors [94]. PER-1 enzyme was detected from *Pseudomonas aeruginosa* and *Salmonella* isolates in Turkey [90]. Its gene has been recorded at least in three different plasmids [90].

Strains producing these enzymes are resistant to penicillins, all cephalosporins and aztreonam, and are susceptible to carbapenems and cephamycins. PER-1 enzyme confers high resistance to ceftazidime, susceptibility to ceftazidime-clavulanate and a reduced susceptibility to piperacillin [90–92].

Carbapenemases. There are four enzymes with carbapenemase activity: Sme-1 [45, 95], Imi-1 [43, 96], NMC-A [94, 100] and IMP-1 [95]. Sme-1, Imi-1 and NMC-A are class A enzymes, all of which are encoded by nontransferable chromosomal inserts. These enzymes have been described in isolates of *Bacteroides* spp., *Stenotrophomonas maltophilia*, *Aeromonas hydrophila*, *Flavobacterium odoratum*, *Bacillus cereus*,

Enterobacter cloacae and *S. marcescens* [94, 98–100]. They are resistant to penicillins, aztreonam and carbapenems. Enzymes are subject to inhibition by clavulanate [14].

IMP-1 enzyme is also carbapenemase, but belongs to molecular class B, this is a metalloenzyme. IMP-1 enzyme was described in three *P. aeruginosa* [101] and five *S. marcescens* isolates in Japan [102]. It was encoded by a plasmid and was transferable among these species. They were not reversed by any known inhibitor. The strains were susceptible only to aztreonam. The enzyme, because of its molecular structure, was inhibited by EDTA and heavy metal ions [14].

Plasmid-mediated cephalosporinases. Since 1989, more and more plasmid-borne cephalosporinases were described. They are belonging to the molecular class B. These enzymes are MIR-1 [16], CMY-2 [103], FEC-1 [17], FPM-1 [104], BIL-1 [18, 19, 62], LAT-1 [105], and MOX-1 [15, 106]. These plasmid determined enzymes are very similar to chromosomal cephalosporinase, encoded by *ampC* gene. It seems that these genes escaped on extrachromosomal elements, and encode even broader-spectrum beta-lactamases [106]. The gene encoding for MIR-1 and BIL-1 is related to the *ampC* gene from *E. cloacae* [16], CMY-2 has a strong homology with *ampC* gene from *C. freundii* [94], FPM-1 with *ampC* gene from *P. mirabilis* [104], and MOX-1 with *ampC* gene from *P. aeruginosa* [106]. These enzymes have been described usually in *E. coli* and in *Klebsiella* spp, except of FPM-1 enzyme, which has been reported in *P. mirabilis* [14]. They can hydrolyse all beta-lactams except carbapenems. These enzymes cannot be reversed by sulbactam or clavulanate, except FEC-1 and MOX-1 enzymes [14].

Oxacillinases. OXA-11 enzyme has been reported to have extended spectrum activity [42]. This enzyme is belonging to the molecular class D. It was isolated from *P. aeruginosa* in a hospital in Ankara. OXA-11 [88] is OXA-10's extended spectrum mutant and resistant to cefotaxime, ceftriaxone, aztreonam and ceftazidime. OXA-11 is inactive to carbapenems and is poorly inhibited by clavulanate [39, 42, 44, 107].

Conclusion

In summary, the vast majority of ESBLs are the derivatives of TEM and SHV-enzymes. There is a great variation in the prevalence of resistance determinants among strains of hospital intensive care units. Bacteria possessing extended-spectrum, plasmid mediated beta-lactamases have been described world-wide and recognized to disseminate locally. The most important are those of *K. pneumoniae* isolates, being difficult to eradicate once they have entered a hospital. Use of third-generation cephalosporins or aztreonam is an important risk factor, and there is associated resistance to other antimicrobials. While the overall incidence of ESBLs has been low, the major concern is that these plasmid-mediated enzymes will spread during the forthcoming years as their predecessors have over the previous two decades [108–110].

The prevention must be in focus. The dissemination of 7-oxyimino cephalosporins in clinical use caused the emergence of newer ESBLs. It is very important to control the

antibiotic treatment, to give up the monolithic use of the third generation cephalosporins. While strains producing ESBLs having importance in nosocomial outbreaks, the change in antibiotic policy in hospitals could control them. The infection control can decrease the prevalence through the strict antibiotic policy and isolation.

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CLINICAL, LABORATORY, AND SEROLOGICAL FINDINGS OF ADULT HUNGARIAN HOSPITALIZED ACUTE HEPATITIS PATIENTS, AND POSSIBLE SOURCE OF THE INFECTION

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Clinical, epidemiological features of acute viral hepatitis of 331 hospitalized adult patients were evaluated. HA, HB, HC, and non A-C H were diagnosed in 36.6%, 34.1%, 10.6%, and 18.7%, respectively. Age of HA cases was significantly lower than that of other cases. Only HA showed seasonal variation. Acquisition of HA was often associated with visits in endemic areas when compared with all other types, while HB, HC, and non A-C H were rather associated with iatrogenic events (blood transfusion, surgical procedures, and hospitalization).

Symptoms of fever and diarrhea, and high ESR were more frequent in HA than in other types, while signs of weight loss and high levels of ALT, AST, and S.T.B, and decreased PT index were significantly more frequent in HB. Cholestasis course was found in 1.7%, 0.9%, and 3.2% of patients with HA, HB, and non A-C H, respectively. Fulminant course was found only in 0.9% of HB patients. Factors as sex and age had no effect on severity of acute phase in HA, HC, and non A-C H, while only the age of patients was inversely associated with severity of acute phase in H B.

Viral hepatitis is probably the world's commonest clinical disease caused by viruses. The infection is unquestionably ancient, it backs to Hippocrates' time. The great development in the diagnosis of viral hepatitis and availability of sensitive tests for different types of acute viral hepatitis have greatly improved our knowledge on the epidemiological and clinical features of the disease.

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This study has been carried out in order to characterize the current epidemiological clinical, laboratory and comparative findings for different types of acute viral hepatitis in hospitalized adults patients.

Patients and Methods

Patients. The study included all the adult patients with clinical, biochemical, and serological evidence of acute viral hepatitis, who had consecutively admitted to adult departments in Szent László hospital, from 1st January 1993 to 1st July 1994.

Methods. The diagnostic criteria for enrollment in the study were based on:

1. Patient's age ≥ 14 years; 2. Recent onset of symptoms and signs of acute viral hepatitis; 3. Rise of aminotransferase enzymes to five times of normal value in hepatitis A, and B, and to two and a half times of normal values in hepatitis C, and non A-C; 4. Positivity of corresponding serological test for each type of acute viral hepatitis; 5. Exclusion of chronic liver diseases and other possible causes of liver damage

"Cholestatic course" was applied to infection with acute viral hepatitis, which had a prolonged severe jaundice with pruritus accompanied by high elevated ALP lasting for > 3 months.

"Fulminant course" was applied to infection with acute viral hepatitis of less than 4 weeks duration before development of hepatic failure with grades III and IV coma according to the criteria of Trey and Davidson [1].

Patterns of acute phase of viral hepatitis were classified according to the description of Dienstag [2] as follows:

"Severe course": ALT peak > 2000 U/I; "Moderate course": ALT peak between 800–2000 U/I; "Mild course": ALT peak < 800 U/I.

Serum sampling. Serum samples from patients for biochemical and serological analysis were taken initially on 1st or 2nd day after admission, then weekly during the period of hospitalisation. All sera taken for biochemical testing were separated within 2 hours, and stored at -20°C for the viral serological test.

Biochemical methods. Aminotransferase (ALT and AST), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), and serum total bilirubin (S.T.B) assays were performed by standard techniques.

Hematological methods. Haemoglobin (Hb), white cell count (WBC), lymphocyte percent (Ly%), platelet count (PLT), erythrocyte sedimentation rate (ESR), and prothrombin index (PT) were performed at least one time by using the routine methods, and by the following criteria: Hb < 90 g/l was assessed as anemia, WBC < 2000 cells/ μl was assessed as leukocytopenia, Ly% $> 40\%$ of WBC was assessed as lymphocytosis, PLT $< 100.000/\mu\text{l}$ was assessed as thrombocytopenia.

ESR was assessed elevated, if it was > 15 mm/h for patients of age 20–50 years, > 20 mm/h for patient of age < 20 years, and > 50 years, PT $< 70\%$ of control prothrombin time was assessed as prolonged.

Serological methods. Acute hepatitis A was defined by the presence of anti-HAV IgM, by the use of Organon Teknika Hapanostika and Roche Cobas-Core HAV IgM tests.

Acute hepatitis B was defined by the concomitant presence of HBsAg and anti-HBc IgM or anti-HBc IgM alone, by use of Roche Cobas-Core, Automated HBsAg, and Organon Teknika Hapanostika, and Uniform II. HBeAg and corresponding antibody (anti-HBe) were measured in some patients by the use of Organon Teknika Hapanostika.

Hepatitis D was defined by the presence of anti-HDV IgG by the use of anti-HDV IgG Organon Teknika Hapanostika test and in anti-HDV IgG positive patients anti-HDV IgM by using anti-HDV IgM

Murex or Sanofi tests. Anti-HBc IgM with HBsAg or without HBsAg test were used to differentiate between the co-infection and superinfection forms.

Acute hepatitis C was defined by detection of anti-HCV IgG by the use of second generation ELISA (Ortho and Ubi tests), and it was confirmed by ratio of Optical Density/Cut Off (OD/CO) > 2. and by exclusion of HAV, HBV, and cytomegalovirus (CMV), Epstein-Barr virus (EBV), toxoplasmosis, and leptospirosis infections.

Non A-C hepatitis was diagnosed if there was no serological evidence of acute hepatitis A, B, C, and other likely causes of acute viral hepatitis.

Anamnesis. All patients were asked to complete a self administered questionnaire, containing, in addition to demographic and clinical data, questions concerning known and putative source of infection in the previous six months, like profession, household exposure to persons with a history of hepatitis, sexual intercourse with partners at risk, parenteral drug addiction, alcohol consumption, dental treatment, hospitalization, surgery, parenteral treatment or blood transfusion, travel abroad or at home. Personal interviews were also performed.

Statistical analysis. The Chi-Square test and Student's test were used. A P value of 0.05 or less was considered as statistically significant.

Results

Out of total 331 patients who had classified as acute viral hepatitis 172 (52%) were males and 159 (48%) were females, with a mean age of 40.6 ± 19.1 years (Range 14–90).

The etiology of classified acute viral hepatitis in 331 patients was as follows: 121 patients (36.6%) had acute hepatitis A, 113 patients (34.1%) had acute hepatitis B, 35 patients (10.6%) had acute hepatitis C, and 62 patients (18.7%) had acute non A-C hepatitis.

Hepatitis A: 121 patients (36.6%) were provisionally classified. One of them was positive for HBsAg but negative for anti-HBcAg IgM, and was considered to be HbsAg carrier with acute hepatitis A virus superinfection. Hepatitis B: 113 patients (34.1%) were provisionally classified, in 107 (94.7%) of them by the concomitant presence of HBsAg and anti-HBcAg IgM, and in 6 (5.3%) by presence of anti-HBcAg IgM without HBsAg. Hepatitis D: None of the patients had serological evidence of acute HDV infection neither co-infection, nor superinfection with HBV infection in the study period. Hepatitis C: Out of 35 patients who were positive to anti-HCV test, 32 (92.4%) were positive during the acute phase of the infection, while the remaining 3 (8.6%) seroconverted during the follow-up period, at 3rd month after admission. Non A-C Hepatitis: The 62 patients who were negative for anti-HCV antibodies, the test was repeated at 3rd month, and at 6th month after the admission.

As shown in Table I, HA patients were significantly younger than HB, HC, and non A-C H patients ($p < 0.00001$), while HB patients were somewhat younger than HC and non A-C H patients ($p < 0.05$ and $p < 0.02$, respectively). The number of female patients were significantly higher among HB patients than among HA, HC and non A-C H patients ($p < 0.01$, $p < 0.03$ and $p < 0.01$, respectively).

Table I

Etiology and demographic features of acute viral hepatitis in 331 patients

	No. of patients		Males/Females	Mean age (yrs±SD*)	Range
	no.	%			
Hepatitis A	121**	36.6	71/50	(28.6±10.3) ^b	14–70
Hepatitis B	113***	34.1	42/71 ^a	(44.2±20.7) ^c	15–90
Hepatitis C	35****	10.6	20/15	(52.1±17.7)	26–86
Hepatitis non A-C	62	18.7	39/23	(51.7±17.1)	16–89
Total	331	100	172/159	40.6±19.1	14–90

* standard deviation

** include one HBsAg carrier

*** 6 cases (5.3%) of them were classified by presence of anti-HBcAg IgM alone

**** 3 cases (8.6%) of them were classified at 3 months after admission

^a $p < 0.001$ vs. HAV, $p < 0.03$ vs. HC, $p < 0.01$ vs. non A-C H^b $p < 0.00001$ in all, vs. HB, vs. HC, and vs. non A-C H^c $p < 0.05$ vs. HC, $p < 0.02$ vs. non A-C H

Age and sex distribution. As shown in Table II, the age distribution seems to be different according to type of acute viral hepatitis. Hepatitis A was more predominant in the young age groups, incidence of HB showed two peaks in 20–29 years age group and in ≥ 60 years age group, while incidence of HC and non A-C hepatitides, increased dramatically with increasing age if compared with hepatitis A and B.

Seasonal variation. As shown in Table III, the incidence of total cases regardless of hepatitis type was almost uniformly distributed through different seasons during the study period. According to type of hepatitis, the incidence seems to be also similar during the different seasons. Only one statistical difference was found, namely, the incidence of hepatitis A was significantly higher in autumn than in winter and spring ($p < 0.04$ and $p < 0.005$, respectively); and in summer than in spring ($p < 0.02$).

Source of infection. Probable sources of different types of acute viral hepatitis are shown in Table IV.

Hepatitis A was significantly more frequently associated with history of secondary contact when compared with C and non A-C hepatitides ($p < 0.03$ and $p < 0.005$, respectively); with history of visit of endemic area when compared with B, C and non A-C hepatitides, ($p < 0.00005$, $p < 0.0008$ and $p < 0.0001$, respectively); and with history of community life contact when compared with B hepatitis, $p < 0.01$.

Hepatitis B was significantly more frequently associated with history of blood transfusion, surgical procedure, and hospitalization, and professional exposure when compared with hepatitis A, ($p < 0.0007$, $p < 0.006$, $p < 0.01$ and $p < 0.005$, respectively). Hepatitis C was significantly more often associated with blood transfusion when compared with A and B hepatitides, ($p < 0.00001$ and $p < 0.003$, respectively), with

Table II

Age and sex distribution of 331 patients with different types of acute viral hepatitis

	HA patients			HB patients			HC patients			Non A-C H patients			Total		
	No.	M/F	(%)	No.	M/F	(%)	No.	M/F	(%)	No.	M/F	(%)	No.	M/F	(%)
14-19 yrs	25	20/5	(20.7) ^a	6	4/2	(5.3)	0	0	(0)	1	0/1	(1.7)	32	24/8	(9.7)
20-29 yrs	47	29/18	(38.8) ^b	34	14/20	(30.1) ^c	5	2/3	(14.3)	8	3/5	(12.9)	94	48/46	(28.4)
30-39 yrs	36	17/19	(29.7) ^d	18	5/13	(15.9)	6	3/3	(17.4)	8	3/5	(12.9)	68	28/40	(20.5)
40-49 yrs	10	4/6	(8.3)	8	1/7	(7.1)	7	3/4	(20.7) ^e	11	7/4	(17.7) ^f	36	15/21	(10.9)
50-59 yrs	1	0/1	(0.8) ^g	15	6/9	(13.3)	5	3/2	(14.3)	11	9/2	(17.7)	32	18/14	(9.7)
≥ 60 yrs	2	1/1	(1.7) ^h	32	12/20	(28.3)	12	9/3	(34.3)	23	17/6	(37.1)	69	39/30	(20.8)
Total	121	71/50	(100)	113	42/71	(100)	35	20/15	(100)	62	39/23	(100)	331	172/159	(100)

^a $p < 0.0005$ vs. HB, $p < 0.003$ vs. HC, $p < 0.0004$ vs. non A-C H^b $p < 0.006$ vs. HC, $p < 0.0002$ vs. non A-C H^c $p < 0.01$ vs. non A-C H^d $p < 0.01$ in both vs. HB and vs. non A-C H^e $p < 0.04$ vs HA, $p < 0.02$ vs. HB^f $p < 0.03$ vs. HB^g $p < 0.0001$ vs. HB, $p < 0.0002$ vs. HC, $p < 0.00001$ vs. non A-C H^h $p < 0.00001$ in all vs. HB, vs. HC and vs. non A-C H

history of hospitalization when compared with A and B hepatitides, and with history of surgical procedure when compared with A, B, and non A-C hepatitides ($p < 0.00001$, $p < 0.001$ and $p < 0.02$, respectively).

Non A-C hepatitis was significantly more frequently associated with blood transfusion, surgical procedure, and hospitalization when compared with A hepatitis ($p < 0.0003$, $p < 0.0003$ and $p < 0.0001$, respectively).

Clinical features. Clinical features of different types of hepatitis are shown in Tables V and VI. Mean duration of hospitalization of hepatitis B patients was longer than that of patients with A, C, and non A-C hepatitides ($p < 0.00001$, $p < 0.05$ and $p < 0.02$), and non A-C and C patients spent longer periods in hospital when compared with patients with hepatitis A ($p < 0.00001$ in both).

Jaundice was more frequent than other symptoms in all types of hepatitis, it was more frequent in hepatitis A than in C and non A-C hepatitides ($p < 0.04$ and $p < 0.00004$, respectively), and in hepatitis B than in C and non A-C hepatitides ($p < 0.00008$ and $p < 0.00001$, respectively). Nausea/vomiting were significantly more frequent in hepatitis A than in C and non A-C hepatitides ($p < 0.002$ and $p < 0.004$, respectively). Fever/chill occurred more frequently in hepatitis A than in B, C and non A-C hepatitides ($p < 0.00001$, $p < 0.00001$ and $p < 0.004$, respectively), and in hepatitis non A-C than in B and C hepatitides ($p < 0.01$ and $p < 0.0006$, respectively). Diarrhea was significantly more frequent in hepatitis A than in B, C, and non A-C hepatitides ($p < 0.0006$, $p < 0.004$ and $p < 0.01$, respectively). Arthralgia/myalgia were more frequent in hepatitis B than in A, and C hepatitides ($p < 0.04$ and $p < 0.01$, respectively). Weight loss was significantly more frequent in hepatitis B than in A, C, and non A-C hepatitides ($p < 0.01$, $p < 0.03$ and $p < 0.03$, respectively). Frequency of atypical course in different types is shown in Table V.

Table III

Seasonal distribution of patients with different types of acute viral hepatitis

	Winter		Spring		Summer		Autumn		Total	
	no.	(%)	no.	(%)	no.	(%)	no.	(%)	no.	(%)
HA patients	18	(20.9)	14	(16.3)	24	(27.9) ^a	30	(34.9) ^b	86	(100)
HB patients	20	(24.7)	17	(21)	24	(29.6)	20	(24.7)	81	(100)
HC patients	5	(20)	9	(36)	6	(24)	5	(20)	25	(100)
Non A-C H patients	7	(20.6)	11	(32.3)	9	(26.5)	7	(20.6)	34	(100)
Total	50	(22.1)	51	(22.6)	63	(27.9)	62	(27.4)	226	(100)

^a $p < 0.02$ vs. spring

^b $p < 0.04$ vs. winter, $p < 0.005$ vs. spring

Table IV

Probable source of infection in patients with different types of acute viral hepatitis

	HA (n=121)		HB (n=113)		HC (n=35)		Non A-C H (n=62)		All subjects (n=331)	
	no.	(%)	no.	(%)	no.	(%)	no.	(%)	no.	(%)
Blood transfusion	0	(0)	5	(4.4) ^a	7	(20) ^b	6	(9.7) ^a	18	(5.4)
Surgical procedure	1	(0.8)	9	(8) ^a	10	(28.5) ^c	8	(12) ^a	28	(8.5)
Hospitalization	5	(4.1)	20	(17.7) ^a	7	(20) ^b	14	(22.5) ^a	46	(13.9)
Hemodialysis	0	(0)	1	(0.9)	0	(0)	1	(1.6)	2	(0.6)
Dental treatment	3	(2.5)	4	(3.5)	2	(5.7)	1	(1.6)	10	(3)
Secondary contact	14	(11.6) ^d	6	(5.3)	0	(0)	0	(0)	20	(6)
Professional exposure	4	(3.3)	15	(13.2) ^a	1	(2.9)	4	(6.5)	24	(7.3)
Intravenous drug abuse	0	(0)	1	(0.9)	1	(2.9)	0	(0)	2	(0.6)
Homosexual	0	(0)	2	(1.8)	0	(0)	0	(0)	2	(0.6)
Community life contact	11	(9.1) ^e	2	(1.8)	0	(0)	2	(3.2)	15	(4.5)
Visit of endemic area	31	(25.6) ^f	7	(6.2)	0	(0)	2	(3.2)	40	(12.1)
Unknown	52	(43)	41	(36.3)	7	(20)	24	(38.7)	124	(37.5)
Total	121	(100)	113	(100)	35	(100)	62	(100)	331	(100)

* School, university, jail

^a significant only when compared with HA^b significant only when compared with HA, and HB^c significant when compared with HA, HB, and non A-C H^d significant only when compared with HC, and non A-C H^e significant only when compared with HB^f significant when compared with HB, HC, and non A-C H

Laboratory findings. Results of liver function tests in different types of acute viral hepatitis are shown in Table VII. Values of ALT, AST, and S.T.B in hepatitis B were significantly higher than in A, C and non A-C hepatitides ($p < 0.00001$, $p < 0.00001$ and $p < 0.0001$, respectively). Values of ALT in hepatitis A were significantly higher than in C, and non A-C hepatitides ($p < 0.0005$ and $p < 0.0001$, respectively). Values of ALP in hepatitis A were significantly higher than in non A-C hepatitis ($p < 0.05$). Values of GGT in hepatitis C were significantly higher than in A and B hepatitides ($p < 0.02$).

Results of some hematological findings in different types of acute viral hepatitis are shown in Table VIII. High ESR was significantly more frequent in hepatitis A than in

B, C, and non A-C hepatitis, ($p < 0.00001$, $p < 0.001$ and $p < 0.00002$, respectively). Decreased PT index was significantly more frequent in hepatitis B than in A, C, and non A-C hepatitis ($p < 0.0002$, $p < 0.05$ and $p < 0.01$, respectively).

Table V

Clinical features of acute phase in different types of acute viral hepatitis

	Time of hospitalization mean (Dys) $\pm$ SD*	No. of symptomatic cases		No. of cholestatic cases		No. of fulminant cases	
		No.	(%)	No.	(%)	No.	(%)
HA patients (n = 121)	(13.7 $\pm$ 5.7)	118	(97.5) ^d	2	(1.7)	0	(0)
HB patients (n = 113)	(24.6 $\pm$ 13.7) ^a	111	(98.2) ^e	1	(0.9)	1	(0.9)
HC patients (n = 35)	(18.7 $\pm$ 8.2) ^b	30	(85.7)	0	(0)	0	(0)
Non A-C H patients (n = 62)	(19.5 $\pm$ 10.6) ^c	54	(87.1)	2	(3.2)	0	(0)

* standard deviation

^a $p < 0.00001$ vs. HA, $p < 0.05$ vs. HC, $p < 0.02$ vs. non A-C H

^b $p < 0.00001$ vs. HA

^c $p < 0.00001$ vs. HA

^d $p < 0.005$ in both, vs. HC and vs. non A-C H

^e $p < 0.002$ in both, vs. HC and vs. non A-C H

Distribution of types of acute phase and effect of sex and age factors in different types of acute viral hepatitis. As shown in Table IX, there were significant differences in frequency of types of acute phase among different types of acute viral hepatitis. Mild course was more frequent in hepatitis C than in A and B hepatitis ($p < 0.01$ and $p < 0.0002$, respectively), and in non A-C hepatitis than in A, and B hepatitis ($p < 0.02$ and $p < 0.0003$, respectively). Severe course was significantly more frequent in hepatitis B than in A, C, and non A-C hepatitis ($p < 0.01$, $p < 0.00002$ and $p < 0.00001$, respectively), and in hepatitis A than in C, and non A-C hepatitis ($p < 0.003$ and $p < 0.002$, respectively). No statistical differences were found in sex distribution among types of acute phase.

The differences in mean age according to type of acute phase were not significant in any types of acute viral hepatitis, except, in HB, the patients of mild acute phase were significantly older than patients of moderate, and severe acute phase ($p < 0.01$ and $p < 0.0001$, respectively). The patients of moderate acute phases were older than patients of severe acute phase ($p < 0.02$).

Table VI

Frequency of occurrence of symptoms and signs in different types of acute viral hepatitis

	Hepatitis A (n = 121)		Hepatitis B (n = 113)		Hepatitis C (n = 35)		Non A-C hepatitis (n = 62)	
Jaundice								
no. (%)	115	(95) ^a	111	(98.2) ^a	28	(80)	46	(74.1)
Nausea/vomiting								
no. (%)	83	(68.6) ^a	58	(51.3)	14	(40)	29	(46.8)
Dark urine/clay-colored stool								
no. (%)	82	(67.8)	74	(65.5)	19	(54.2)	33	(53.2)
Fever/chills								
no. (%)	72	(59.5) ^b	22	(19.4)	2	(5.7)	23	(37.1) ^c
Anorexia								
no. (%)	67	(55.4)	49	(43.3)	13	(37.1)	28	(45.1)
Abdominal pain/discomfort								
no. (%)	64	(52.9)	63	(55.8)	13	(37.1)	28	(45.1)
Fatigue/malaise								
no. (%)	31	(25.6)	33	(29.2)	10	(28.6)	24	(38.7)
Diarrhea								
no. (%)	19	(15.7) ^b	3	(2.7)	1	(2.9)	2	(3.2)
Influenza like syndrome								
no. (%)	15	(12.4)	9	(8)	3	(8.6)	7	(11.3)
Arthralgia/myalgia								
no. (%)	13	(10.7)	23	(20.4) ^d	1	(2.9)	9	(14.6)
Headache								
no. (%)	9	(7.4)	5	(4.4)	1	(2.9)	3	(4.8)
Pruritus								
no. (%)	7	(5.8)	8	(7.1)	5	(14.3)	3	(4.8)
Dizziness								
no. (%)	2	(1.7)	4	(3.6)	2	(5.7)	1	(1.6)
Hepatomegaly								
no. (%)	108	(89.2)	99	(87.6)	28	(80)	53	(85.5)
Splenomegaly								
no. (%)	37	(30.6)	20	(17.7)	5	(14.3)	10	(16.2)
Weight loss								
no. (%)	25	(20.6)	37	(32.7) ^e	5	(14.3)	11	(17.7)

^a significant only when compared with HC and non A-C H^b significant when compared with HB, HC and non A-C H^c significant only when compared with HB and HC^d significant only when compared with HA and HC^e significant when compared with HA, HC and non A-C H

Table VII

Results of liver function tests in different types of acute viral hepatitis

	Hepatitis A (n = 120)*	Hepatitis B (n = 112)*	Hepatitis C (n = 35)	Non A-C hepatitis (n = 62)
Mean maximum ALT (U/l) $\pm$ SD**	(1651.7 $\pm$ 1032) ^a	(1968.6 $\pm$ 1149.3) ^b	(957.9 $\pm$ 622.8)	(1052.5 $\pm$ 766.9)
Mean maximum AST (U/l) $\pm$ SD**	(1024.9 $\pm$ 941.6)	(1540.2 $\pm$ 981.1) ^c	(785.5 $\pm$ 502.2)	(874.1 $\pm$ 838.6)
Mean maximum ALP (U/l) $\pm$ SD**	(574.8 $\pm$ 281.1) ^d	(519.1 $\pm$ 232.8)	(484.8 $\pm$ 230.9)	(482.5 $\pm$ 252.9)
Mean maximum GGT (U/l) $\pm$ SD**	(258.8 $\pm$ 203.6)	(253.1 $\pm$ 195.6)	(354.1 $\pm$ 242) ^e	(314.4 $\pm$ 272.8)
Mean maximum S.T.B (μ mol/l) $\pm$ SD**	(139.2 $\pm$ 79)	(223 $\pm$ 127.3) ^f	(146.7 $\pm$ 127.9)	(147.7 $\pm$ 139.1)

* results of liver function tests for one pregnant was not included

** standard deviation

^a p < 0.0005 vs. HC, p < 0.0001 vs. non A-C H^b p < 0.05 vs. HA, p < 0.00001 vs. HC, p < 0.00001 vs. non A-C H^c p < 0.00002 vs. HA, p < 0.00001 vs. HC, p < 0.00001 vs. non A-C H^d p < 0.05 vs. non A-C H^e p < 0.02 in both, vs. HA and HB^f p < 0.00001 vs. HA, p < 0.001 vs. HC, p < 0.0001 vs. non A-C H

Discussion

In the last decades, incidence of acute viral hepatitis decreased in Hungary [3, 4], due to improvement of sanitation level, hygienic conditions, and socioeconomic status, besides screening of blood donors and availability of passive and active immunization. In light of these events adult population becomes susceptible to a clinically more severe and debilitating viral hepatitis infection.

In the present study HA was the most frequent type of acute viral hepatitis similar to other observations from the Central Eastern European region [5]. Distribution of HA was not equal in the different age groups: the incidence was high in younger patients (< 30 years), and it was rare in older patients ($\geq$ 60 years). This means that acute HA is adolescent's and young adult's disease due to the gradual improvement of the hygienic conditions in the country.

Table VIII

Frequency of some hematological findings in different types of acute viral hepatitis

	Hb < 90 g/l		WBC < 2×10 ⁹ /l		LY > 40% of WBC		PTL < 100×10 ⁹ /l		ESR > normal rate		PT < 70% of index	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
Hepatitis A (n = 120)*	0	(0)	0	(0)	6	(5)	1	(0.8)	66	(55) ^a	22	(18.3)
Hepatitis B (n = 110)*	1	(0.9)	0	(0)	12	(10.9)	3	(2.7)	19	(17.3)	44	(40) ^b
Hepatitis C (n = 34)*	1	(2.9)	0	(0)	2	(5.8)	1	(2.9)	8	(23.5)	7	(20.6)
Non A-C hepatitis (n = 57)*	1	(1.8)	0	(0)	4	(7)	1	(1.8)	12	(21.1)	11	(19.2)

* Pregnants, and patients who had anticoagulant treatment were not included

^a p < 0.00001 vs. HB, p < 0.001 vs. HC, p < 0.00002 vs. non A-C H^b p < 0.0002 vs. HA, p < 0.05 vs. HC, p < 0.01 vs. non A-C H

The seasonal variation in HA incidence proved to be similar to other observations [4]. This is related to summer holidays, and visit of endemic areas, if we take in account that 25.6% of HA patients had traveled to endemic areas during the summer. The frequency of HB was the second among different types of acute viral hepatitis, it was more frequent in older patients than HA, and showed two peaks in its incidence, like in other reports from Central-Eastern Europe [6], the first peak may be related to heavy exposure to the infection by sexual contacts or by professional exposure, the second peak may be related to heavy exposure to iatrogenic factors.

Negative result of HBsAg at admission in 5.3% of HB patients coincides with some reports by others [7], which reflect that some patients probably can clear HBsAg at the early stage of acute phase.

No recorded HDV infection, either by co-infection or by superinfection form with HBV is consistent with the low prevalence of HDV carriers in chronic HBV infection in Hungary [8, 18]. NANB Hepatitis was the third in frequency among different types of acute viral hepatitis. It is possible that some patients had chronic HCV infection, when enrolled in the present study, because the presence of anti-HCV IgG does not distinguish acute from chronic HCV infection, features of the infection, however, were generally consistent with criteria of diagnosis of acute viral hepatitis.

Incidence of C hepatitis increased consistently with increase of age, it was absent in young patients (< 20 years), but increased to highest levels in older patients (≥ 60 years). This finding contrasts to other recent reports [9], that have somewhat younger patients with a greater number of drug abusers.

Table IX

Distribution of types of acute phase in different types of acute viral hepatitis

	Mild course (Mx. ** ALT < 800 U/l)				Moderate course (Mx. ** ALT = 800–2000 U/l)				Severe course (Mx. ** ALT > 2000 U/l)				Total			
	No.	M/F	(%)	mean age (yrs)±SD*	No.	M/F	(%)	mean age (yrs)±SD*	No.	M/F	(%)	mean age (yrs)±SD*	No.	M/F	(%)	mean age (yrs)±SD*
Hepatitis A (n = 121)	27	14/13	(22.3)	30.4±13	58	34/24	(47.9)	27.2±10.6	36	23/13	(29.8) ^e	29.4±7.1	121	71/50	(100)	28.6±10.3
Hepatitis B (n = 113)	18	9/9	(15.9)	63.5±8.3 ^a	44	14/30	(38.9)	45.7±23.8 ^b	51	19/32	(45.2) ^f	36±15.7	113	42/71	(100)	44.2±20.7
Hepatitis C (n = 35)	16	10/6	(45.7) ^c	56.3±16.5	17	10/7	(48.6)	50.2±18.5	2	0/2	(5.7)	33±5.7	35	20/15	(100)	52.1±17.7
Non A-C hepatitis (n = 62)	25	17/8	(40.3) ^d	51.2±14.8	31	19/12	(50)	52.5±18.8	6	3/3	(9.7)	49.8±20.2	62	39/23	(100)	51.7±17.1

* standard deviation

** maximum

^a p < 0.01 vs. moderate, p < 0.0001 vs. severe^b p < 0.02 vs. severe^c p < 0.01 vs. HA, p < 0.0002 vs. HB^d p < 0.02 vs. HA, p < 0.0003 vs. HB^e p < 0.003 vs. HC, p < 0.002 vs. non A-C H^f p < 0.01 vs. HA, p < 0.00002 vs. HC, p < 0.00001 vs. non A-C H

Non A-C hepatitis plays a main role in NANB hepatitis in Hungary like in recent reports from various parts of the world [10, 11].

In the present study, the cause of non A-C hepatitis in some cases may be attributed to HCV infection, as use of PCR in diagnosis of acute HCV infection has a limited benefit. This benefit has been estimated in some studies at 1.6–12% as additional rate in diagnosis by ELISA II [9, 12].

Some cases of non A-C hepatitis may be attributed to HEV, but the number of the cases seems to be small, although the prevalence of HEV seropositivity in Europe can be 3 to 10 percent in some regions [13, 46].

It is clear from the foregoing discussion that the etiological agent in the majority of non A-C hepatitis is probably not HCV or HEV, but other agent or agents. This finding is confirmed by other recent reports [14–18].

Non A-C hepatitis seemed to be more frequent in older patients similar to C hepatitis, without significant differences between mean age of HC and non A-C H patients. This finding contrasts to other reports, because the majority of C hepatitis patients in other reports – in contrast to the present study – consisted of young drug abusers [9, 12, 19].

Acquisition of hepatitis A seems to be more often associated with anamnestic history of visits to endemic areas, in addition to secondary and community life contacts than that of other types of hepatitis [20].

Despite screening of all blood donors for HBV by sensitive tests in Hungary [21], post-transfusion HBV infection was reported in the present study similar to other reports [22], as to the blood may contain sufficient HBV DNA to be infective, but too small amounts of HBsAg. Anti-HBcAg antibodies are not tested. Iatrogenic factors (surgery, hospitalization, hemodialysis, and dental treatment) and professional exposure were frequent in the present study as risk factors in acquisition of B hepatitis similar to other studies [8]. Male homosexuality and intravenous drug abuse were reported as risk factors in acquisition of HB, but these seem to be less important in Hungary than in Western European countries [19, 23].

Due to screening of blood donors by anti-HCV test, post-transfusion NANB hepatitis have been decreased in Hungary [24], nevertheless post-transfusion NANB hepatitis (C and non A-C) occurred in some blood recipients similar to the observation of an other study [25]. Iatrogenic factors (blood transfusion, surgery, and hospitalization) were main potential sources of C and non A-C hepatitis, too. Other risk factors were shown to be similar to those described in an other study [26], except some differences in the surgical procedures, which were significantly more often associated with HC than with non A-C H [27]. Risk factors of community life contact and visit to endemic areas were more often associated with acquisition of non A-C hepatitis than with C hepatitis. This finding supports the view that non A-C hepatitis agent may spread by other than parenteral routes.

As in HB, intravenous drug abuse seemed to be rarely associated with HC and non A-C hepatitis, in contrast to reports from Western European countries, and from the USA [19, 28, 29]. This may reflect the low number of intravenous drug abusers in Hungary, which may be related to strict legal or economic pressures. The source of infection remained undefined in about 20–43% of patients with different types of acute viral

hepatitis. This may be related to poor memory of patients or to contact with subclinical cases or healthy carriers.

Clinical features. The rate of symptomatic acute viral hepatitis was high. In accordance with previous observations the clinical symptoms of A and B hepatitis were more severe than those of C and non A-C hepatitis [2, 30, 31]. The most frequent symptoms were: jaundice in both HA and HB [2, 9, 12, 30, 31], nausea and vomiting in HA [32], fever and chill in both HA and non A-C H [33], diarrhea in HA [30], arthralgia, myalgia, and loss of weight in HB [34], levels of ALT, AST, and S.T.B. in HB [29, 31]; ALT and ALP levels in HA [29, 31]; levels of GGT in HC were higher than in diseases of other etiology [35].

In general, frequency of anemia, leukopenia, relative lymphocytosis, and thrombocytopenia were low and similar to those seen among different types of acute viral hepatitis [36], nevertheless high ESR and decreased PT index were more frequent in HA, and in HB, respectively [36, 37]. Distinct correlation was found between the age and the severity of acute phase in HB [38]. Incidence of fulminant acute viral hepatitis was very low. Only one single case of HB has been found [39–41].

Inconsistent with other published data, cholestatic pattern was not more frequent in HA compared with other types [42, 43], and non A-C hepatitis was not milder than HC [12]. This may be related to the differences in source of infection, or to differences in mean age of patients, or perhaps to different genotypes of HCV, which may affect the severity of acute phase of HCV infection [44, 45].

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Abbreviations

HA: Hepatitis A, HAV: Hepatitis A virus, Anti-HAV: Antibody against A virus, Anti-HAV IgM: Antibody against A virus of IgM type, HB: Hepatitis B, HBsAg: Hepatitis B surface antigen, Anti-HBs: Antibody against hepatitis B surface antigen, HBcAg: Hepatitis B core antigen, Anti-HBcAg: Antibody against hepatitis B core antigen, Anti-HBcAg IgM: Antibody against hepatitis B core antigen of IgM type, HBeAg: Hepatitis B e antigen, Anti-HBe: Antibody against hepatitis B e antigen, HD: Hepatitis D, HDV: Hepatitis D virus, Anti-HDV: Antibody against hepatitis D virus, HC: Hepatitis C, HCV: Hepatitis C virus, HE: Hepatitis E, HEV: Hepatitis E virus, NANBH: Non-A, non-B hepatitis, Non A-CH: Non-A, non-B hepatitis which is negative to HCV serological test, ELISA: Enzyme linked immunosorbent assay, PCR: Polymerase chain reaction, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, GGT: Gamma-glutamyl transferase, S.T.B: Serum total bilirubin, ESR: Erythrocyte sedimentation rate Hb: Haemoglobin, WBC: White blood cell count, Ly: Lymphocyte count, PT: Prothrombin index.

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COMPACT GROWTH OF *STAPHYLOCOCCUS HAEMOLYTICUS* IN SOFT AGAR IS NOT DUE TO HYDROPHOBIC INTERACTION BETWEEN THE COCCI

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It was hypothesized that the formation of compact colony in soft-agar both in the presence and absence of serum, characteristic mainly for strains of the species *Staphylococcus haemolyticus* among coagulase-negative staphylococci [Szűcs et al. *Acta Microbiologica Hungarica* 40:181–189 (1993)] was due to hydrophobic interaction between cocci. The effect of a number of surface active agents on this phenomenon was examined. Neither 0.1% and 1% Tween-80 nor 5% and 10% ethylene glycol and polyethylene glycol nor 0.1%–4% trypsin influenced the colony morphology in soft-agar prepared in modified *Staphylococcus* 110 broth. Bovine lactoferrin and apolactoferrin at concentrations of 0.1%–0.4% made compact colonies transient to diffuse ones. Thus, cocci are not adhered to each other in compact ball-like colonies by hydrophobic interaction or trypsin-sensitive proteins. It is possible that still unknown polysaccharide-binding proteins or other trypsin-resistant proteins are responsible for the formation of compact colonies by *Staphylococcus haemolyticus* in soft-agar.

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Growing part of Gram-positive infections have been caused by coagulase-negative staphylococci (CNS). Diseases caused by these bacteria have mainly appeared as nosocomial infections: sinusitis paranasalis, endocarditis, septicaemia, osteomyelitis, infections of urogenital and respiratory tracts [1–3]. This wide spreading of CNS is partly due to the fact that more and more biomaterials (such as artificial valves, hip joints, catheters, cannulae, etc.) have been recently applied in invasive diagnostical and therapeutical inventions. CNS have strong affinity to these plastics which serve as a surface of adhesion and colonisation for them [4–6]. It would be a great result if we could solve the problem what factors can cause this ability. Bacterial adhesion to artificial and native surfaces – so each other – occur mainly by hydrophobic and electrostatical interactions. Besides, biospecific receptor-ligand bond could take part, too [5, 7–11].

Our examinations are based on a previously established fact, that different species of CNS form different type of colonies in soft-agar (SA) based on modified Staphylococcus broth. The colony morphology can be compact, transient and diffuse type. These colonies likely have different degree of internal cohesive strength [11–13]. Szűcs et al. have recently observed that the majority of the strains of *Staphylococcus haemolyticus* form compact colonies in SA, while *Staphylococcus epidermidis* forms only diffuse colonies [13].

This study addresses the question what factors can cause the close adhesion between cocci in compact colonies: hydrophobic interaction of membranes, cell-surface proteins or others. According to literature data Tweens, ethylene-glycol and trypsin – although in different ways – reduce drastically cell-surface hydrophobicity [9–11, 14]. We have supposed that using these agents aggregation would be suspended, so cocci would be detached from each other and colony formation would start from separated bacteria. Since a surface active agent in a growth medium has an effect during the whole period of bacterial multiplication, the morphology of appearing colonies might be different from those ones growing in non-supplemented medium. Accordingly, the aim of this study was to answer the question whether hydrophobic interaction cause cohesion of cocci to each other. Therefore, we have examined the effect of some surface active agents such as Tween-80, ethylene glycol, polyethylene glycol, trypsin, bovine lactoferrin and apolactoferrin on growth properties of *S. haemolyticus* in soft-agar.

Materials and methods

Sources of strains. The five wild-type *S. haemolyticus* strains included in this study were isolated from patients treated in our clinic. Two of them were isolated from urine (strains 9117, 17101), one from pus of wound (strain 9381), secretion of pharynx (strain 16789) and one came from pus of pyoderma (strain 17462). Taxonomic identification was performed according to a combined scheme from Kloos and Schleifer [15], and Akatov et al. [16]. The strains were stored in dextrose-broth at –20 °C containing 25% (v/v) glycerol. Reference strains were *S. haemolyticus* (HNCMB 110013), *S. epidermidis* (HNCMB 110012) and *S. aureus* (Smith diffuse) [17].

Growth media. The bovine blood agar, glucose-meat broth, modified Staphylococcus medium 110 (mS 110) broth (Difco Laboratories, Detroit, Michigan, USA), and soft-agar (SA) media used for different cultivations were previously described in details [13].

Surface active agents to diminish cell surface hydrophobicity. Polyethylene-glycol 400 (PEG, Biogal, Debrecen, Hungary), ethylene-glycol (EG, Reanal, Budapest, Hungary) and Tween-80 (Reanal) as detergents were used, respectively. Trypsin was used to digest proteins attached to cell surfaces. Besides, the effects of bovine lactoferrin (Lf) and apolactoferrin (apo-Lf) on colony morphology were studied. Lf was kindly donated by Dr. A.S. Naidu [18].

Proceeding of experiments. Strains were inoculated from glycerol broth on blood agar plates and incubated for 24 h at 37 °C. From blood agar cultures of each strain – that proved pure – a loopful bacteria was inoculated into 5 ml 1% dextrose-broth and incubated overnight at 37 °C. These cultures were then diluted in physiological saline solution up to 10^{-6} and 0.1 ml of 10^{-4} and 10^{-6} dilution was added to prepared growth media in tubes.

These prepared media were: (1) mS-110 broth for control; (2) SA (mS-110 broth with 0.15% Noble-agar) for control; (3) SA containing PEG (5%, 10% and 20%); (4) SA containing EG (10% and 20%); (5) SA containing Tween 80 (0.1% and 1%); (6) SA containing trypsin (1%, 2% and 4%); (7) SA containing Lf (0.1%, 0.2% and 0.4%); (8) SA containing apo-Lf (0.1%, 0.2% and 0.4%).

After thorough mixing incubation lasted for 24 h and 48 h at 37 °C. Each experiment was carried out at least three times.

Examination of colony morphology. Extent and shape of colonies was estimated with naked eye. Colony morphology in SA was categorized into three groups: a ball-like "compact", an elongated condense-band-like "diffuse" and a so called "transient" form – which was between the former ones possessing a head and a hanging tail. "Mixed" growth meant that more types of colonies appeared in one tube.

Results

Growth properties in the presence of surface active agents. Control cultures of all strains in mS broth free of agar did not form isolated colonies but a viscous, filamentous deposit appeared at the bottom of tubes. Above this deposit broth was clear. In SA all of the *S. haemolyticus* strains formed compact colonies, one of the strains (strain 9381) grew in mixed form (compact-transient). Reference *S. haemolyticus* strain grew in compact colony as well. Tween-80 in concentrations of 0.1% and 1% had not effect on colony morphology since colonies appeared in the original form showed in SA. The effects of PEG and EG were studied at 5%, 10% and 20% concentrations. In our experiments growth of *S. haemolyticus* strains were partially prevented by EG at 10% and totally at 20%. Colony change also did not occur below 10% concentration of EG, only a few transient colonies appeared among compact ones in one case. There was not seen considerable difference between effects of EG and PEG.

Growth properties in the presence of lactoferrin and apolactoferrin. Tables I and II show the colony formation in the presence of Lf and apo-Lf. Almost all of the strains responded with a change in colony form to the presence of Lf and apo-Lf. At concentrations 0.1%–0.2% of Lf compact colonies turned to transient ones. Few colonies turned to diffuse ones at 0.4% of Lf. Besides, the colony number was fewer at higher Lf concentrations. Changes appeared stronger in the presence of apo-Lf. Diffuse forms were produced at lower concentrations and the shape of transient types were elongated, likewise diffuse ones. It was interesting that size of colonies became smaller (contrary to

results of trypsin-supplementation), for example, compact colonies contracted as tiny as a pin-point. Neither *S. epidermidis* nor *S. aureus* reference strains showed significant changes in diffuse growth.

Table I

*Colony formation of Staphylococcus haemolyticus strains
in the presence of bovine lactoferrin in soft-agar*

<i>Staphylococcus</i> strains	In SA	In 0.1% Lf-SA	In 0.2% Lf-SA	In 0.4% Lf-SA
<i>S. aureus</i> (Smith diffuse)	D	D	D	D
<i>S. epidermidis</i> HNCMB 110012	D	D	D	D
<i>S. haemolyticus</i> HNCMB 110013	C,T	D,T	D,T	D,T
<i>S. haemolyticus</i> (9117)	C	D,T	T	T
<i>S. haemolyticus</i> (9381)	C	C,T	T	T
<i>S. haemolyticus</i> (16789)	C	C	C,T	D,T
<i>S. haemolyticus</i> (17101)	C	T	T	D,T
<i>S. haemolyticus</i> (17462)	C	C	T	T

Lf: lactoferrin

T: transient colony

C: compact colony

C,T: compact-transient (mixed) growth

D: diffuse colony

D,T: diffuse-transient (mixed) growth

Table II

*Colony formation of Staphylococcus haemolyticus strains
in the presence of bovine apolactoferrin in soft-agar*

<i>Staphylococcus</i> strains	In SA	In 0.1% Alf-SA	In 0.2% Alf-SA	In 0.4% Alf-SA
<i>S. aureus</i> (Smith diffuse)	D	D	D	D
<i>S. epidermidis</i> HNCMB 110012	D	D	D	D
<i>S. haemolyticus</i> HNCMB 110013	C,T	D,T	D,T	D,T
<i>S. haemolyticus</i> (9117)	C	C,T	C,T	D,T
<i>S. haemolyticus</i> (9381)	C	T	T	D,T
<i>S. haemolyticus</i> (16789)	C	C	C,T	D,T
<i>S. haemolyticus</i> (17101)	C	T	T	T
<i>S. haemolyticus</i> (17462)	C	C,T	T	D,T

Alf: apolactoferrin

D: diffuse colony

C: compact colony

C,T: compact-transient (mixed) growth

T: transient colony

D,T: diffuse-transient (mixed) growth

Discussion

The pathomechanism of CNS infections is far from a full understanding, however it is certain that beginning steps – such as adhesion and colonisation – play important roles [1–3, 12]. Bacteria can attach to native and artificial surfaces – so each other – by really the same aspecific and specific bonds. The aspecific interactions might be of electrostatic or hydrophobic nature [5, 7, 8, 11, 12]. Since generally both bacterial cell surface and the attached cell (or artificial object) surface has a net negative charge, so there will rise an electrostatic repulsion. This repulsion can be overcome by hydrophobic interactions. During the process of adhesion, when hydrophobic bonds have been established, the surface protruding groups involved in the bond come so close to each other that short-range forces such as van der Waals forces, hydrogen bonds may become operative [9]. Biospecific bonds affect specifically between a cell surface receptor and its ligand. These receptors can be virulence factors, for example collagen binding protein and fibronectin binding protein in case of staphylococci [2, 3, 9, 12]. On the basis of the formers it is evident that virulence of a bacterium depends considerably on the adhesial ability determined by its own cell surface structure.

In the course of our examinations we could conclude from the structural changes of colony morphology of *S. haemolyticus* strains caused by surface active agents to the forces affecting inside of a colony. According to the results of some authors the cell surface hydrophobicity is the factor which forms the compact type colonies in SA [5, 7–9, 11, 14]. To eliminate cell surface hydrophobicity we used surface active agents (Tween-80, ethylene-glycol, polyethylene-glycol) and to prevent interactions of cell surface proteins we used trypsin in order to digest them.

On the basis of our results we can state that compact colony formation which is a strong characteristic of *S. haemolyticus* strains is due to neither cell surface hydrophobicity nor interactions of trypsin-sensitive cell surface proteins. Growth in the presence of detergents (Tween-80, ethylene-glycol, polyethylene-glycol) fails to change colony morphology. Using trypsin both compact and diffuse colonies have abundantly grown, but shape of colonies have not changed at all. Abundant growth can be explained that this enzyme – wanted originally to use for digestion of cell surface proteins – could also be utilized by the cocci as nutriment.

Other part of our experiments referred to the colony morphology changing under the effects of Lf and apo-Lf. The effect of bovine and human Lf and apo-Lf as natural antimicrobial drugs have been examined in vivo and in vitro by several researches. The expected results have not been obtained, only a weak “protective” effect has appeared which seemed to be due to aspecific macrophage activation. On the other hand, both Lf and apo-Lf could prevent multiplication of bacteria in vitro, and apo-Lf exerted a stronger effect [19, 21].

There are two theories to interpret this phenomenon. The first explanation refers to the competition between microorganisms and ironchelating compounds, since ferro ions found in growth media (or in organisms) are essential for bacterial respiration [20]. The second theory assumes porins responsible for the inhibiting effect. These so-called porins are hydrophilic protein channels that were found in the outer membrane of Gram-negative

bacteria. These channels can make a gate to cross the outer membrane for small and hydrophilic compounds but a barrier for large and hydrophobic molecules. Porins are specific Lf-binding receptors, too [22]. It was first proved at a *Salmonella hartford* strain that the presence of Lf on porin channels changed permeability of membranes and increased sensitivity of bacteria to antibiotics [23]. Since then the existence of the same receptors have been proved in *S. aureus* strains. In the course of our examinations we tried to get an answer whether the proved membrane activity of Lf and apo-Lf can affect cell-surface binding conditions so colony formation in SA. Our results demonstrate that these two natural iron chelating proteins change the form of *S. haemolyticus* colonies indeed, so compact types elongate and become transient and diffuse type in SA, assuming that Lf and apo-Lf are able to rearrange or to change cell-surface properties. The analysis in which way it happens and what factors are responsible for forming compact colonies indeed (polysaccharide-binding components, trypsin-resistant proteins, etc.) can serve as a subject of another study.

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GROWTH OF MICROORGANISMS ON RUBBERS AND PLASTICS. MEDICAL ASPECTS OF MICROBIOLOGICAL CORROSION AND THE ROLE OF QUALITY CONTROL *

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The cause of nosocomial infections and their possible solutions, according to the chemical compounds of rubber and plastic equipments are shortly summarized. Compounds of rubbers and plastics act as culture medium for bacteria and fungi being causative agents in nosocomial infections and because of their long-term persistence they can be source of such infections.

This problem needs cooperation between experts and specialists from different scientific fields working in both industry and medicine. Only "united forces" can make steps in order to find solutions to these emerging questions and problems.

Rubbers and plastics are organic materials. According to their extended and wide use, irreversible damages caused by microorganisms like fungi and bacteria have been observed first on military and electrotechnical fields.

In order to prevent microbiological corrosion a Designation has been issued first by the proposal of the American Society for Testing Materials (ASTM) [1], followed by an International Organisation for Standardisation (ISO) recommendation for standards [2]. These provided the base of standards in Hungary; first for the study of resistance to moulds [3], then resistance to both moulds and bacteria [4, 5] and finally the standards for plastics and rubbers used in medicine [6].

All study standards suggest a bacterium species, *Pseudomonas aeruginosa* – one of the leading causative agent in nosocomial and iatrogenic infections – to be the test microorganism in examinations of the resistance of plastics and rubbers against microbiological corrosion.

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Important to point out that Hungarian literature dealt with this bacterium very detailed as cause of nosocomial infections [7], but said nothing about its role in microbiological corrosion.

Experiments in our Institute proved that many plastic and rubber materials due to their chemical compounds supplied nutrients for growth of microbes and allowed long-term persistence. Furthermore, the invasion of microorganisms by positive chemotaxis seemed to be enhanced. Therefore, a closed correlation was showed between microbiological corrosion and nosocomial infections [8].

The principal of the above-mentioned studies of standards was the following. The test rubber or plastic material was examined as a carbon source in the presence of NH_4NO_3 as a nitrogen source in 50 ml minimal nutrient base and based on an increase in germ number, the rubber/plastic material was qualified as attackable, moderate attackable and resistant material, respectively. When the germ number exceeded $2 \times 10^8/\text{ml}$ it was considered attackable, moderate attackable when the N_{max} was between $10^7/\text{ml}$ – $2 \times 10^8/\text{ml}$ and resistant when the germ count did not exceeded $10^7/\text{ml}$ [9, 10].

In our experiments it was observed that in the case of high germ number, further bacteria, mainly other Gram-negative rods – which have also played role in nosocomial infections – could survive in the presence of *P. aeruginosa* (unpublished data).

Those monomers (softeners, emollients, stabilizers, enhancers, sliders etc.) which are used in the manufacturing of plastics and rubbers, may be responsible for and support microbial growth.

Our results of recent studies have established that first of all the aliphatic emollients e.g. dioctyl-sebateate, were the easiest to destruct and utilize by microorganisms. Furthermore, it was shown that among many Gram-negative bacteria, only *P. aeruginosa* was able to utilize dioctyl-sebateate within 5 days in the presence of NH_4NO_3 . Among stabilizers, Zn-stearate increased highly the germ number already in low concentration (unpublished data).

It is known that these mentioned additional compounds used in plastic and rubber industry can also be destructed by fungi, like *Candida albicans* and other yeasts, and by pancreatin enzyme from higher eucariotic organisms. According to the data, showing that wide range of microbes play role in microbiological corrosion, nowadays study standards and proposals exists for so-called resistant and sensitive monomers and polymers [11–15].

Inhibitory additional compounds, so-called biocides (not allowing the growth of bacteria and fungi), can have protective effect only if the polimer-monomer construction is made of materials being not available for microbes. Biocide, being effective against *P. aeruginosa* has not been published yet [16].

In case of plastic products there is a correlation between the germ number and the detected/measured weight loss in the materials. Furthermore, macroscopic alterations could be observed on the plastics, like discoloring, rigidity and contraction.

In our summing up study using the rules of the Hungarian medical standard MSZ 03-154-84, we gained the following experiences and results:

The generally used rubber bed sheets (Hungarian Standard No.: MSZ-03-107-79) tested with *P. aeruginosa* became macroscopically mouldy and medium attackable against bacteria (germ number $10^8/\text{ml}$). The weight loss of the sample was 0.9 mg/cm^2 during the 28 days of examination.

Among medical equipment the ear plugs of the machines for hard and dull hearers and the different infusion systems resulted to be attackable. We could culture *P. aeruginosa* from "sterile" inside of intubation tubes and drains as well.

Among nursing equipment certain plastic sheets resulted to be highly attackable. Ulmer and Saratoga drains used in kidney transplantation were attackable, too.

Rubbers used and sold in pharmaceutical praxis were attackable, e.g. the rubber cap in eye droppers. Medium attackable were the plastic covers and (bottle) caps. Oil contamination rendered all samples to be attackable (unpublished data).

In dental surgery the base plate soaked in sunflower oil and the Isopast® filling became attackable, Evicrol [9] and other so-called photopolimeric fillings resulted to be resistant [10].

Industrial data: We found samples among wrapping material (original packing made for direct use in food industry) which were contaminated with *P. aeruginosa* [17]. Furthermore, among milking machine rubbers only one type, called "Mélotte" was resistant. Samples of other types predisposed garget (udder mastitis) by *P. aeruginosa* in cows [18]. In workers, working in press, upper respiratory tract infections occurred after a while, due to airconditioning in the press, because biofilm developed in the moisturising system of the aircondition machine and that was spread around in the air in form of aeroplanktons [19].

It is well known that nosocomial infections are still an emerging problem, demanding arrangements [20].

A positive and a negative list of materials should be set up and study standard examinations should be obligatory as well.

More support is needed in basic research and innovation in order to develop high resistant compounds against microbes, for the industry.

The trend of reuse of disposable equipment (tubes, drains), the above-mentioned problems of microbiological corrosion should be taken in consideration more intense.

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A NEW SELECTIVE METHOD FOR ISOLATION OF *HAEMOPHILUS* SPECIES

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Chocolate agar with teicoplanin disk (30 µg) was used for the isolation of *Haemophilus* strains. Fifty strains of 3 *Haemophilus* species grew as well in the inhibition zones of teicoplanin disks as on teicoplanin containing selective plates, whereas Gram-positive bacteria failed to form colonies. This selective method proved especially advantageous when *Haemophilus* strains were isolated from mixed bacterial cultures of 665 specimens.

Strains of *Haemophilus* genus are frequent pathogens of the middle ear and respiratory infections. Their aetiological role is especially significant in acute otitis media and chronic bronchitis [1, 2]. Isolation of a pathogenic agent and determination of its antibiotic sensitivity pattern is a basic principle of adequate antibiotic treatment. Isolation of *Haemophilus* strains from specimens of the upper respiratory tract is a difficult task because the mixed flora overgrows the *Haemophilus* colonies.

In contrast, *Haemophilus* strains are relatively resistant to teicoplanin, whereas Gram-positive bacteria show great sensitivity to this antibiotic [3, 4]. Therefore, teicoplanin was found suitable for the selective isolation of *Haemophilus* strains.

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Materials and methods

Strains. Fifty *Haemophilus* strains were studied; they were cultured from hospitalized patients with otitis, laryngitis, bronchitis and vaginitis. The strains were isolated on chocolate agar.

Species determination of the strains was performed by Sim's growth factor requirement method as well as on the basis of haemolysis, sucrose and xylose decomposition, indole production and ONPG test [5]. Distribution of the 50 *Haemophilus* strains were as follows: 30 *H. influenzae*, 18 *H. parainfluenzae*, and 2 *H. parahaemolyticus*.

Determination of teicoplanin sensitivity. (1) *Disk method:* paper disks of 30 µg teicoplanin (Unipath, Oxoid, Ltd. Basingstoke, Hampshire, UK) were placed on the chocolate agar swabbed densely with bacterial suspension in physiological saline and incubated for 24 h at 37 °C. (2) *Plate dilution method:* physiological saline suspensions of the strains were seeded in sectors on chocolate agar plates containing 120, 60, 30 and 15 µg/ml teicoplanin (teicoplanin sodium. Bulk. C.A. N° 95002378) and on teicoplanin free chocolate agar as control and incubated for 24 h. (3) *Examination of the isolated Haemophilus colonies.* Of the dilutions of 5 *Haemophilus* strains suspended in physiological saline 0.05 ml aliquots were placed on chocolate agar containing 120 µg/ml teicoplanin as well as on control plates. The number of the colonies and their morphology on selective and non-selective chocolate agar plates were compared.

Examination of selective effect of teicoplanin on 665 specimens: 263 pharyngeal or laryngeal discharges, 279 sputum, 35 ear puses, 88 vaginal excretions were seeded on surface of chocolate and blood agar plates with teicoplanin disks and on a Sabouraud slants.

Composition of chocolate agar. Nutrient agar was prepared from beef infusion containing 2% agar and 1% Witte peptone, pH 7.4 and subsequently supplemented with 5% bovine blood at 80 °C and the bottles were left to stand at the same temperature for 30 min.

Results

Haemophilus strains showed a relative resistance to teicoplanin. Paper disks of 30 µg teicoplanin failed to show an inhibition zone on 50 *Haemophilus* cultures. As to colony morphology, abundant growth was observed with all strains with plate dilution method, using 120, 60, 30 and 15 µg/ml teicoplanin concentrations. This finding was confirmed by examination of colony counts on selective and non-selective chocolate agar plates.

The advantage of the selective plates were found striking when 665 clinical specimens were examined. Thirty-two *Haemophilus* strains were isolated from 665 specimens. The species distribution of the strains were: 24 *H. influenzae*, 7 *H. parainfluenzae* and 1 *H. parahaemolyticus*. Bacteria of normal pharyngeal mucus belong to Gram-positive microorganisms in their majority failed to form colonies in the inhibition zone of teicoplanin disks even after 48 h incubation. Sometimes colonies of *Neisseria subflava*, *Neisseria mucosa*, *Enterococcus faecalis*, species of *Enterobacteriaceae* and *Candida albicans* were seen in the inhibition zones. However, the presence or absence of colonies of these species on chocolate and blood agar plates or Sabouraud slants make easy the differentiation in the teicoplanin inhibition zones, too.

Discussion

Teicoplanin is considered to be ineffective on *Haemophilus* species [6, 7]. In our study we could demonstrate similar resistance of *Haemophilus* strains to teicoplanin. Gram-positive aerobe and microaerophil bacteria showed a high sensitivity to teicoplanin in vitro [8–20]. Table I summarized the minimal inhibition concentrations of teicoplanin for different microorganisms according references [8–20].

Table I

In vitro sensitivity of various microorganisms to teicoplanin according references

Species	Teicoplanin minimal inhibitory concentration (µg/ml)
<i>Staphylococcus aureus</i>	0.5–2.0
Coagulase negative <i>Staphylococcus</i>	0.1–8.0
<i>Streptococcus pyogenes</i>	2.0
<i>Streptococcus mitis</i>	0.2
<i>Enterococcus faecalis</i>	0.2–3.1
<i>Propionibacterium acnes</i>	0.4
JK diphteroid	0.8
<i>Listeria monocytogenes</i>	0.06

In our clinical study of 665 specimens were subjected to the selective effect of teicoplanin. We could also demonstrate strong antimicrobial effect of teicoplanin on Gram-positive bacteria. Sometimes we saw teicoplanin resistant colony in teicoplanin inhibition zone. Moderate sensitive or resistant *Enterococcus faecalis* strains were isolated by Vincent et al. [18], Williamson et al. [19]. Our examination confirmed the strong activity of teicoplanin against Gram-positive bacteria. The excellent selective effect of teicoplanin and the resistance of *Haemophilus* made this antibiotic suitable for selective isolation of the *Haemophilus* species from clinical materials containing a predominantly Gram-positive flora. The results of parallel seeding specimens on chocolate agar plates with and without teicoplanin disks showed that not only the rate of *Haemophilus* positive results grew by 20% but the identification and antibiotic sensitivity determination of colonies isolated on selective plates could be carried out more rapidly and simply.

Isolation of *H. influenzae* from materials containing Gram-positive bacteria (respiratory tract, ear, eye and genital organs) is a difficult task. Turk and May's method of washing the sputum with physiological saline reduces *Haemophilus* count together with the accompanying flora [1]. Füzi described a chocolate agar with 25 µg/ml vancomycin content which gave excellent results in the isolation of *H. influenzae* [21]. Baber supplemented the chocolate agar with bacitracin at 10 µg/ml concentration [22].

Crawford et al. prepared bacitracin-containing chocolate agar with rabbit blood for distinguishing the haemolytic strains [23]. Little [24] used a combination of bacitracin with crystal violet and Hansen et al. with cloxacillin for selection [25].

Chocolate agar with teicoplanin disk was found effective in rapid isolation of *Haemophilus* strains from materials contaminated with Gram-positive bacteria and provided quick and reliable assistance in the diagnosis of *Haemophilus* infections.

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BACTERIAL ENDOTOXINS AND NONSPECIFIC RESISTANCE*

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The stress situations, including medical interventions (e.g. operations, antitumor drugs, irradiation, etc.) decrease the nonspecific resistance of the body. In these situations patients people have greater chance to get an opportunistic infection than healthy ones. The restoration or elevation of the activity of immune system in injured patients is a very important task of medicine. Minute amounts of bacterial endotoxin (LPS) – given parenterally – can elevate the nonspecific resistance. Unfortunately this beneficial influence is associated with noxious properties. Irradiation (60 Co-gamma; 150 kGy) is a good technique for the detoxification of LPS. The radiodetoxified endotoxin (RD-LPS) preparation (so-called TOLERIN) is less toxic but its beneficial properties is preserved. On the basis of animal experiments and clinical trials TOLERIN could be a suitable preparation for regeneration of the lymphoreticular-immune system and elevation of nonspecific resistance.

The classical immunology is concerned with specificity (e.g. antigen-antibody reaction). Although nonspecific reactions are very important in host defense this has not been in the centre of interest of immunologists [1]. However, it was discovered that in AIDS the decrease of nonspecific resistance is one of the most important cause of the deadly outcome. This elevated the interest of immunologists in the natural resistance. The situation is similar in the case of seriously ill cancer patients. The medical interventions (e.g. operations, antitumor drugs, irradiation) decrease the nonspecific resistance of the patients. So these patients have much greater chance to get an opportunistic infection than healthy people. The restoration or elevation of the activity of immune system in injured patients will become a very important task for immunologists. The enhancement of natural defense or stimulation of nonspecific resistance is one of the most important unsolved problems of medicine today [2]. Many authors have observed that within 24

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hours of intravenous inoculation with killed typhoid bacilli, increased resistance to the live culture developed in animals [3]. The cell walls of certain Gram-negative bacteria were also found to be particularly active in stimulating this nonspecific resistance. Other workers identified this active material as a lipopolysaccharide, so-called endotoxin (LPS) [4, 5]. The effects of this material upon resistance have been demonstrated in infections induced by parasites, fungi, gram-positive/gram-negative bacteria, mycobacteria and viruses [6]. It is also well known that repeated parenteral injections of small doses of LPS will produce so-called endotoxin tolerance [7]. The endotoxin tolerance can inhibit the release of ribonuclease which is induced by toxic dose of endotoxin [8]. The most important property of bacterial LPS is probably its capacity to stimulate the body's natural defense. Thus, in an immunologically nonspecific manner, the different endotoxin shocks, induced by gram-negative bacteria or LPSs may be prevented. Unfortunately, this beneficial influence is associated with some noxious properties (e.g. pyrogenic, hypotensive, abortifacient etc.). This is why such LPS are not suitable for the enhancing of nonspecific resistance in endotoxin-sensitive mammalian species, including humans. For this reason, to decrease the lethality of LPS while retaining the beneficial properties has become the aim of number of researchers. Various techniques (physical, chemical etc.) have been used for detoxification with varying results. Perhaps one of the best detoxification technique is the use of ionizing radiation [9–11].

Irradiation of LPS with 60-Co (150 kGy) decreased its noxious effects significantly in a dose-dependent manner. The radiodetoxified (RD-LPS) preparations were less active than the parent (toxic) LPS in inducing various other undesirable responses. However, RD-LPS (so-called TOLERIN) preserved the beneficial effects of LPS, as summarized in earlier publications [2, 12, 13]. These findings are confirmed by other authors, too [14, 15]. Irradiation modifies markedly the chemical structure of LPS, decreases the amount of certain constituents e.g. glucosamine, KDO, fatty acids etc. [14, 16, 17].

A single parenteral injection of RD-LPS can prevent the experimental endotoxin shock in all investigated mammals. Eighty-four per cent of dogs survived the hemorrhagic shock, and 90% of rats survived the fecal septic peritonitis when they were pretreated with RD-LPS. Such pretreatment may be useful in the preparation of gastrointestinal surgery. The RD-LPS protected 74% of rats with experimental intestinal ischemia induced by superior mesenteric artery occlusion, too, and 60 per cent of pretreated rats survived tourniquet shock: experimental limb ischemia. The RD-LPS pretreatment prevented the abortifacient effect of LPS (90%). Moreover, the RD-LPS has membrane-stabilizing effect and hereby can prevent the membrane-damaging effect of LPS and some cytostatics. The cardiovascular action of endotoxin is well known in various forms of shock. However, the RD-LPS has barely any hypotensive effect. The RD-LPS pretreatment can prevent practically all hemodynamic changes induced by LPS. LPS plays an important role in the pathogenesis of the so-called intestinal syndrome of radiations disease, too. However, the RD-LPS pretreatment saved 70% of the irradiated rats. Moreover the RD-LPS pretreatment can prevent the effect of LPS induced oxydative stress in glutathion redox system and can moderate the level of hypoxic metabolites (lactic and uric acids) and severity of acidosis (Boda et al., unpublished data, 1988). LPS

is well known to exert a marked adjuvant activity on the immune response of mammals. The RD-LPS preparation retained this adjuvant activity.

Perhaps one of the most important effects of RD-LPS is the influence on regeneration of lymphoreticular-immune system. It is well known that ionizing radiation decreases all immune functions. We have found that the immune response against sheep red blood cells of sublethally irradiated (7.0 Gy, 60-Co-gamma) rats was regenerated by treatment with a single dose of RD-LPS on day 21. The relative radioresistant T-helper cell population has an important role in this regenerating effect of RD-LPS. The RD-LPS can evoke the regeneration of immune system in irradiated animals. Decrease of nonspecific resistance in immune deficient or immunosuppressed patients is the most important cause of the opportunistic infections, sepsis, endotoxemia, pneumonia, etc. It is well known that the majority of the patients following organ transplantation die from common septicemia. Antilymphocyte serum (ALS) is a potent immunosuppressant and it was commonly used in organ transplantation. For this reason, the possibility of augmenting of NSR or induction of endotoxin tolerance after ALS treatment has great importance. If RD-LPS was given to ALS-treated rats they became tolerant against the lethal dose of LPS. It is evident that in spite of suppression of T-lymphocytes by ALS, induction of LPS tolerance (enhancement of NSR) was normal. Moreover, the RD-LPS pretreatment can prevent lethal bacterial (*Klebsiella pneumoniae*, *Proteus vulgaris*) infections by elevation of NSR. Facultative pathogen (opportunistic) bacteria may flourish and cause disease when specific and nonspecific resistances are impaired. The lethality of experimental Aujeszky-disease (pseudorabies virus, herpes group) was also reduced by RD-LPS pretreatment from 100% to 30%. In collaboration with Szeri and her coworkers it was demonstrated that RD-LPS can produce significant proliferation of lymphoreticular-immune system in germ-free (practically immune deficient) animals, too [18]. The proliferation of lymphoreticular-immune system by RD-LPS could be also demonstrated in newborn mice. The animals became sensitive against LCM virus, though, the untreated mice survived the infection because their lymphoreticular system (T-lymphocyte function) was insufficient at this age. The RD-LPS preparation preserves many other beneficial effects such as RES and macrophage activation, antitumor activity, etc. [1, 2, 18–20].

The RD-LPS (TOLERIN) has been tested for innocuity in two trials on volunteers in Hungary under the supervision of the Ministry of Health. The first trial was performed in 5 healthy (31–53 years old) volunteers. Seven µg/kg TOLERIN given subcutaneously caused only a temporary weak febrile and local reaction (weak edema and hyperemia). Clinical and laboratory tests showed practically no changes in the parameters investigated except for the increase of the number of white blood cells and complement C₃ fraction at 24 h (Bertók et al., unpublished data, 1987).

In the second trial performed on 40 healthy (18–26 years old) volunteers (university students) we had similar results. The subcutaneously given 4 µg/kg TOLERIN caused only a temporary rise of temperature (1 person) and local reaction (weak hyperemia) in 20 per cent (8 persons) of the volunteers. The values of clinical and laboratory tests were unchanged except for the number of white blood cells and complement C₃ fraction which were elevated within 24 h (Bertók et al., unpublished data, 1988).

On the basis of these results TOLERIN was tested in clinical trials on more than 350 surgical (intestinal tumor bearing), a few immune deficient (AIDS, ARC) and oncological (testis tumor bearing and cisplatin-treated) patients. TOLERIN treatment could prevent the sepsis and activate the bone marrow function.

If the TOLERIN will be registered it could be applied for the enhancement of nonspecific resistance, prevention of endotoxemic shock, regeneration of immune system, complex therapy of cancer and many other aims, too.

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THE ROLE AND IMPORTANCE OF ENDRE HŐGYES IN THE HISTORY OF HUNGARIAN MICROBIOLOGY*

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It was the habit of ancient Romans to mark lucky days' in the calendar with white chalk. In the history of Hungarian microbiology such "dies faustus" is the 30th of November of the year 1847, when Andrew Hőgyes, one of the most brilliant figures of the history of Hungarian medical science, was born.

This year, on the 150th anniversary of his birth, many commemorations have already been made. Thus, I hope not to make a great mistake if I focus only on Hőgyes' microbiological importance.

Hőgyes' carrier started in the Institute for Theoretical Medicine headed by Kálmán Balogh. This was where – probably under the guidance of Balogh himself – his interest turned on microbiology.

His task was to study the then prevailing cholera of still unknown etiology. By animal experiments (1873) he already found that the stool of patients plays a part in the transmission of the disease. However, the experiments had to be ceased because the employee of the institute, whose duty was to collect stool samples, and his little daughter, fell ill with cholera. Luckily they recovered but those who shared a common lavatory with them caught cholera and one of the two severely sick persons died of this illness.

This was the time when Hőgyes was appointed as head of the professorship of pathology at Kolozsvár University and this also contributed to the interruption of his researches. Nevertheless, till the end of his life, Hőgyes became a follower of Pasteur's and Koch's new approach in propagating the pathogenic effect of microbes.

This should be emphasized the more because in those years two prominent authorities of the age, Virchow and Pettenkofer, still were against this idea, and though Semmelweis' (1858) and Lister's (1865) studies were known, doctrinarians published caricatures against Pasteur and loudly criticized the medical science, which obviously' could not achieve anything in the fight against cholera. Especially the voice of homoeopathics was extremely loud – they had great support from the aristocrats who

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preserved feudal traditions and therefore disdained medical doctors. Here we have to notice that 'alternative curing' found a fertile soil even in 19th century America. Hőgyes, however, definitely opposed to these trends. In 1876 in Kolozsvár he founded an Association for Medical and Natural Sciences; in 1882 – as head of an investigation committee – he declared ineffective and thus useless the 'cure-all' remedy of a veterinarian in Kolozsvár against anthrax, and, at the same time, repeatedly called the attention of the respective Minister of State to the outstanding importance of Pasteur's specific vaccination against anthrax. He proclaimed his views in his university lectures, and also explained them in 1882 in a study – published in the Bulletin of Medical and Natural Sciences for the 25th anniversary of the Hungarian Medical Weekly – titled 'Dominating Theories in Hungarian Medical Culture in the Past and Present'. In this study – while giving a survey of the confused situation of his time – he took side for experimental research which was initiated by Czermak and adapted by Kálmán Balogh. At the same time, with great energy, he performed series of experiments. These experiments were primarily the continuations of his neurophysiological examinations started in 1872 as Kálmán Balogh's assistant, and eventually thanks to them and to his subsequent researches carried out on the functioning of the labyrinth and related eye movements that Hőgyes – as an already well-known scientist – was made chairman of the newly established Lyssa Committee. Members of this Committee were – among others – Ákos Azary, Viktor Babes, József Fodor and Ferenc Hutya.

Consequently, his appointment was not mere honour, but appreciation; appreciation of the research work based on experiments that Hőgyes started in less than two weeks after Pasteur's publication on the successful anti-rabies vaccination performed on Joseph Meister and that meant the start of scientific virus research in Hungary.

Hőgyes – since he did not want to stop his experiments – refused being delegated to Paris; Babes went to work in Pasteur's laboratory instead of him. Soon, however, on the 12th August, 1886, an article by Hőgyes could be published in the Hungarian Medical Weekly about producing a fixed strain of rabies virus, first after Pasteur, namely, as second in the world. But it took a long way to get as far as that. As a first step Hőgyes made experiments for virus isolation. He triturated samples got from the brain and different organs of a seven year old boy who died of rabies in Budapest, for inoculating dogs, rabbits, guinea-pigs, mice, then frogs. He made subcutan and subdural inoculations and found that the bulb (medulla oblongata), the kidney and the spleen contain 'infectious material', but the other organs do not. He did not succeed to transfer the virus from sick animals, therefore he was more prudent when using material from an other fatal case. He designed a fixation-frame for the proper fixation of the rabbits' organs to get experimental material for inoculations, and he modified Pasteur's rather tough method for subdural injection and replaced trephine by a dental drill. With this more precise and more reliable method he could make successful serial passages; he confirmed the already existing theories about the identical pathogen of human and animal rabies and proved the possibility of infecting frogs and performing serial passages on frogs.

As a next step, he wanted to determine the most suitable target organ of infection. In the course of his related experiments he found that intraocular injection, or injection through the orbit into the optic nerve, or – even better – through the ethmoid bone into the olfactory bulb was more successful than the subdural inoculation introduced originally by

Pasteur. He inoculated infectious material into the fourth ventricle and the infection was successful if the material was instilled into the sciatic nerve, and especially, if the proximal stump of the transected nerve was infiltrated with his suspension. He examined the effects of s.c., i.m. inoculations and on intact mucous membrane, tympanic membrane, tympanic cavity and on submucos of the tongue and found that the pathogen reaches its main target, the medulla oblongata, at various times from different gates of infection. Based on his findings, Hógyes could already state at this stage that "though we do not know the infectious material of rabies yet, we already know some of its characteristics".

Naturally the main purpose of his experiments was to produce a fixed strain of rabies virus. To get this, Hógyes did not want to use street virus of human origin; he gained his starting material from the medulla oblongata of a dog that was infected by and died of rabies in a natural way. For these important experiments starting on the 28th February, 1886, Hógyes – unlike Pasteur – did not select the rabbits at random, but he consistently chose young animals weighing 800–1000 grams, and he made the passages by using always the brain emulsion of those inoculated rabbits which died in the shortest time. By this genuine method, he achieved that the incubation time of the disease in inoculated rabbits shortened rapidly, in a shorter time than in Pasteur's experiments. After 21 passages Hógyes possessed a fixed virus strain that definitely killed the rabbits in 7 or 7.5 days.

In the study of the biological characteristics of his fixed virus strain Hógyes carefully examined the symptoms of the disease on inoculated rabbits. The recognition of the special temperature curve – what is today called diphasic temperature curve and which is generally considered characteristic for virus-caused encephalomyelitises – is also Hógyes' merit. An other result of these experiments was a successful method used regularly in virus research today without stressing Hógyes' initiative role in inventing it. This method is the biological titration of virus that Hógyes used for the first time in 1888. The first step to this was the emulsification of a piece of the medulla oblongata used for inoculation in physiological salt solution. As we know, Pasteur used bouillon for this purpose. Consequently, it was Hógyes who introduced the use of physiological salt solution into the methodology of virology in 1886 in order to get 10, 100, 250, 500, 1000, 5000 and 10.000 fold dilutions from the brain emulsion used for experimental infections. Based on the inoculations made with the diluted suspension he found that his material definitely kills the rabbits already in a 1000 fold dilution, not consequently in 5000 fold and not at all in a 10000 fold dilution. Therefore we can state that Hógyes was the first to use the method in virus research which resulted in the emergence – though only a few decades later – of the present method of calculating ID_{50} and LD_{50} .

To learn more about the biological characteristics of the pathogen still unknown at that time, Hógyes made comparative research to measure the virulence of his own and Pasteur's fixed strain of rabies virus. In the course of his research he found that neither Pasteur's desiccation method nor his own dilution method weakened – as Pasteur assumed – the virulence of the virus; there is simply less infectious material in the inoculum; namely what we face is not a qualitative but only a quantitative change. At the same time he also found that serial passages made on frogs were able to weaken the virulence of the virus.

Then he started to study the 'protective effect' of his fixed virus strain. Today we would say: he studied its immunogenic effect.

There is no need now to go into details about Hőgyes' antirabic vaccination-method which produced the best results in the world for more than half century and surpassed Pasteur's achievements. However, when we recall his great achievements, we must not forget about the vast experimental work he had done before he used his vaccine on humans. Vaccination of men could only take place after trials on a great number of animal experiments in 1886 and '87 Hőgyes used about 47 dogs for his experiments – after a series of trials proving that the vaccine is harmless and effective.

When judging the oeuvre of a scientist, it is not enough to appreciate the imagination, diligence and endurance he showed in achieving his goals, we also have to consider if he had the special talent to set up a school and to attract valuable collaborators.

Looking at Hőgyes' abilities to create a school – and this time considering exclusively the development of microbiology in Hungary – we should mention first his most noted disciple, Aladár Aujeszky. We also have to mention his associate, József Lőte, who was Hőgyes' main collaborator in his rabies research; further, Tamás Marschalkó who revealed the genesis of plasma cells; Ágoston Székely, Hőgyes' successor as head of the Pasteur Institute in Budapest and Ferenc Tangl, Hőgyes' successor in the university chair, who started his career as a microbiologist.

Besides educating followers, Hőgyes succeeded to attract noted researchers to be his collaborators and it enabled him to enlarge the span of his research and to improve the international fame of Hungarian rabies research. Let me present just a brief list here: on Hőgyes' request Ákos Azary made experiments with dogs; J. Bókai and Ch. Szilágyi studied the tenacity of rabies virus against various chemicals; K. Schaffer, L. Nagy, C. Pándi and A. sarbó studied the neurohistopathology of rabies in men and in animals deceased under natural or experimental conditions; L. Udránszky studied the chemical composition of the central nervous system of healthy dogs, dogs died of rabies and dogs vaccinated against rabies. Remarkable are the clinical surveys of the characteristic symptoms of manifest rabies made by K. Laufenauer, A. Moravcsik and K. Schaffer; Hőgyes himself reported about an unsuccessful hypnotherapeutic experiment made in cooperation with Laufenauer.

Hőgyes regularly reported about the results of all these researches in Hungarian and foreign medical periodicals and books. Consequently, we can say that Hungarian literature on virology was also initiated by Hőgyes.

Beside mentioning Hőgyes' research work which started a new period in the history of microbiology in Hungary, we have to remember his importance as a founder of an institute and hospital, too. Archimedes said; 'Show me a fix point and I will move the world out of its place'. 'Give me a laboratory and I will turn the world out of its corners' – said Pasteur. Hőgyes – recognizing the truth of this saying – also set to the establishment – following the example of the Pasteur Institute in Paris – a Pasteur Institute and Hospital in Budapest. – To see the significance of the job, we should remember that rabies was very frequent in those days in our country and Hungary was on the top of the list concerning the number of human and animal victims; the hospitalization and care for those who developed rabies or were suspected to have it was unorganized and took place in a few, poorly equipped hospitals at their even less suitable psychiatric departments. The

unfortunate patients condemned to death were transferred to the newly opened St. István Hospital only from 1885 on. Apart from emergency hospitals which operated at the time of great epidemics, in this time Hungary had no hospital for epidemics. St. László hospital opened up only in 1894. This date might be of interest, because four years earlier Hógyes had already set up a Pasteur Hospital, which could soon care for 130 inpatients. It is also worthwhile to note that the neuropsychiatric surveys mentioned earlier were made on the patients of the Budapest Pasteur Institute headed by Hógyes.

From the aspect of the history of microbiology in Hungary, we have to focus our attention on the Pasteur Institute of Budapest organized by Hógyes. This is especially important because sometimes this excellent institute for virus research – the third in the world – was mistaken for a Pasteur-Chamberland Laboratory that moved from Vienna to Budapest as a result of a corruption scandal and which supplied Hungary with various vaccines used for curing animals. This market-oriented enterprise of foreign interest had nothing to do with the Pasteur Institute of Budapest which received more patients already in 1896 than that of Paris. However, these two institutes had different fates. The Pasteur Institute in Paris has been developed into an international citadel of microbiology by ROUX, and it served as an example to the establishment of the Koch Institute in Berlin and the Rockefeller Institute in New York. The Pasteur Institute in Paris opened up branches in many countries of the world, and many of the microbiologists working in these institutes obtained Nobel-prizes later. In the Pasteur Institute of Budapest with the tragic disease and death of the founder, Hógyes, the dynamics of research was broken. Although his successor, Ágoston Székely, loyally continued to produce his master's vaccine; in fact there was only stagnation. Later vaccine production was taken over by an other medical institute and – as rabies was eliminated from Hungary –, it meant the elimination of the Pasteur Institute as well; the Budapest Pasteur Institute and Hospital ceased to operate on the 1st January, 1942.

In his life, Endre Hógyes was rewarded with various prizes for his achievements in the fight against rabies and for his other neurophysiological, pathological and other activities. He was a member of and decorated with the Grand Prize the Hungarian Academy of Sciences; his activities were honoured by the king with the title of ministerial counsellor; he was elected member of the National Public Health Council and vice chairman of the Hungarian Royal Association of Natural Sciences. After his death his memory has been preserved by street names, statutes, a decorative tombstone, commemorative medals and a representative monography summarizing the results of his research work.

Nevertheless, there is still need to harmonize our commemorations and I believe that the Hungarian Academy of Sciences is the most competent body in this respect. Now I wanted to throw light especially on Hógyes' microbiological activities. I wanted to call attention to the fact that he was a scientist who was the Hungarian propagator of Pasteur's teachings; who was the first Hungarian virus researcher; who was the first to overcome rabies in Hungary; who was the educator of Hungarian microbiologists and had merits as the founder of a school; who created a marvellous shelter for the Hungarian virus research; who raised the Hungarian research of rabies, or in a wider sense the Hungarian research of zoonosis to an international level; who developed the professional literature of this science and even set out the directions for further development. The task of fostering

Hőgyes' memory should not be restricted to particular societies or to the Hungarian medicine; it should be the task of the entire nation. His name is among the great Hungarians and is still brightly shining.

BOOK RECEIVED

Evolution of microbial life. Edited by D. McL. Roberts, P. Sharp, G. Alderson and M. A. Collins. 54th Symposium of the Society of General Microbiology (University of Warwick). Series editor (1996-2001) Dr. D. Roberst. Cambridge University Press, The Edinburgh Building Shaftesbury Road, Cambridge CB2 2RU, U.K., 1997.
Price: £60.00 (US\$ 115.00) (hardback ISBN: 0 521 56432 8)

A new milestone of microbial evolution has been published in Cambridge in the form of a book of 300 pages. The most significant contribution of the volume to the previously published issues are the recent conclusions drawn on the basis of nucleic acid sequence data. Evolutionary aspects of the host cells, those of autonomous extrachromosomal organelles and elements (i.e. hepatitis C virus, RNA viruses and to a limited extent plasmids, bacteriophages and elements of fungi which do not follow the Mendelian rules of inheritance) are summarized in the proceedings. The evolutionary aspects of the development of metabolic pathways, possible ancient forms of bacteria and eukaryotes are discussed with special attention to cyanobacteria, and anaerobic eukaryotes. Prokaryotic ecto- and endosymbionts which might be both the predecessors of horizontal evolutionary gene transfer, and members of the cascade of events leading from anaerobiosis to facultative anaerobic protists and organisms equipped with mitochondria, chloroplasts and other energy generators of the aerobic cells or specialized symbiotic factories of natural biotechnology are discussed in detail (i.e. Nitrogen fixation, cellulose degradation, hydrogen consumption or production of essential amino acids or vitamins).

Keyword of the whole volume is diversity. Evolution of the diversity of bacteria, quasispecies character of RNA viruses (hepatitis C virus), problems of Eukarya, and those of septation of Fungi. The best conclusion has been cited by L. Dijkhuizen (from Hermes et al., 1990) 'the (evolutionary) process is referred to as in vivo protein engineering'. The whole material published in this volume could be specified as 'biotechnology in nature in order to fulfil requirements of the continuously changing environment of our Globe'. Congratulations to the Editors, and Contributors. The referee is a virologist. According to the opinion of the virologist the role of autonomous extrachromosomal genetic elements in horizontal, and vertical transfer of genetic information is underrepresented in this volume (Gy. Berencsi et al.: Evolutionary aspects, and taxonomic definition of viruses together with mobile extrachromosomal elements of relative autonomy into a special highest rank taxon. *Acta Microbiol. Immunol. Hung.* 42: 141–153, 1995). Nevertheless, this subjective idea does not diminish the value of the outstanding work of Editors and Publishers.

The titles of the individual chapters are the following:

- W. F. Doolittle: Some aspects of the biology of cells and their possible evolutionary significance
J. W. Schopf: Are the oldest fossils cyanobacteria?
S. J. Giovannoni et al.: Ribosomal RNA and the evolution of bacterial diversity
J. A. Lake, M. C. Rivera: The prokaryotic ancestry of eukaryotes
N. W. Gray, D. F. Spencer: Organellar evolution
A. C. Holmes et al.: An RNA virus tree of life?
P. Simmonds: Evolution of hepatitis C virus
M. L. Sogin et al.: Problems with molecular diversity in the Eukarya
T. Fenchel: Eukaryotic life: anaerobic physiology
R. T. Moore: Evolutionary trends in Fungi
A. E. Douglas: Microorganisms in symbiosis: adaptation and specialization
L. Dijkhuizen: Evolution of metabolic pathways
J. P. Gogarten et al.: Gene duplications and horizontal gene transfer during early evolution.

György Berencsi

**ANNUAL MEETING OF THE
HUNGARIAN SOCIETY FOR MICROBIOLOGY**

SZEKSZÁRD, AUGUST 25–27, 1997

ABSTRACTS OF PAPERS AND POSTERS

ANNUAL MEETING OF THE
SOCIETY FOR MICROBIOLOGY

HELD AT THE UNIVERSITY OF CALIFORNIA
AT BERKELEY, CALIFORNIA, AUGUST 22-27, 1957

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PLENARY SESSION

M. PÉTER

Endre Hőgyes and the Transsylvanian Museum Society

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Endre Hőgyes was the head of the Department of General Pathology and Pharmacology of Ferenc József University at Kolozsvár (now Cluj) in 1875–1883. Although in 1859 the Transsylvanian Museum Society (TMS) was established at Kolozsvár, and since 1861 there had been a Department of Natural sciences, too, including physicians as well, one month after his arrival, Hőgyes began to organise the Association of Medical and Natural Sciences. In December 1875, statutory meeting was convened, and later, beginning with the 8th Jan. the *Kolozsvár Association of Medical and Natural Sciences* started its activity with 110 members, and Hőgyes became first its secretary, then its president. The presidency of the TMS disapproved of that, although the Department of Natural Sciences had almost come to a halt at that time. In 1884, the association founded by him by mutual consent joined the Department of Medical and Natural Sciences of TMS, giving a new impulse to its activity.

As early as the year of foundation (1876) Hőgyes started a journal named *Értesítő*, in which the scientific papers presented by the members were published. Beginning with the 4th year (1879), it was edited as the common journal of the Association and TMS, with a number of his articles as well. This publication, after two forced interruptions, is even now alive under the title *Orvostudományi Értesítő* (the 69th volume in 1997), indicating on its cover that it was founded by Endre Hőgyes.

In 1943, the Medical Department of TMS decided that they would organise a festive meeting in his memory every year, here a commemorative medal bearing Endre Hőgyes's name, as well as a national award would be granted to a physician worthy of that. Unfortunately, it happened only once, in 1944.

Although E. Hőgyes spent only 8 years in Transsylvania, he succeeded in making his mark as a scholar in the scientific life there, too.

VIROLOGY AND IMMUNITY

J. SZABÓ, A. BÁCSI, I. ANDIRKÓ, J. KISS, J. NEMES, F. D. TÓTH

Induction of release of infectious human cytomegalovirus from macrophages by coinfection with human T-cell leukemia-lymphoma virus type I

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Human cytomegalovirus (HCMV) infection of macrophages is nonlytic and exclusively cell associated. HCMV is an important pathogen in patients suffering from human T-cell leukemia-lymphoma virus type I (HTLV-I)-associated malignant lymphoproliferative disorders. These observations led us to investigate interactions between HCMV and HTLV-I in M ϕ coinfecting with both viruses. In M ϕ cultures infected with HCMV alone, infectious virus was recovered from M ϕ cellular lysates but not from culture supernatant. In contrast to this finding, dually infected cell cultures released infectious HCMV, reaching a peak between day 5 and 7 post-coinfection. Studies on the expression of HCMV-specific antigens in singly and dually infected M ϕ showed that replication of HCMV was significantly enhanced by coinfecting with HTLV-I. Induction of HCMV release from singly infected cells by transfection of the *tax* gene indicated that HTLV-I Tax protein plays a crucial role in the enhancement of HCMV replication. We further demonstrate that the transactivator gene product of HTLV-I exerted its effect on HCMV both in a cytokine-dependent and independent manner. In conclusion, the results of the present study indicate that the unique adaptation of HCMV to persist in M ϕ can be abolished by coinfecting with HTLV-I, and in vivo dual infection of M ϕ may result in severe complications due to activation of HCMV.

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**Plasma HIV-1 load and disease progression in AIDS patients
of the St. László Hospital**

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Nucleic Acid Sequence Based Amplification (NASBA) is a suitable method for the quantification of HIV-1 RNA in plasma and serum samples. Since determination of the viral load appears to be a valuable marker for the prediction of disease progression and for monitoring the efficiency of antiretroviral therapy, the National AIDS Committee initiated the introduction of NASBA in Hungary at the end of 1996. We obtained plasma samples from patients with pre-AIDS and AIDS of the St. László Hospital, Budapest. In plasma samples of treated or untreated patients with progressive disease we could detect high HIV-1 RNA copy levels (217 000–556 000 copies/ml), while clinically stable patients treated with antiretroviral drugs had relatively low HIV-1 RNA copy numbers (7 400–18 600 copies/ml).

The CD4 T-cell count decreased during progression of the disease, as expected. In general, there was an inverse correlation between CD4 T-cell counts and HIV-1 RNA levels. We concluded that measurement of HIV-1 RNA plasma level has an important role in assessing prognosis and effects of antiretroviral therapy in HIV-infected patients.

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**Genetic subtyping of HIV-1 using heteroduplex mobility
assay (HMA) and genomic sequencing**

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HIV-1 shows remarkable genetic variation which is the result of a high mutation rate and rapid viral turnover. Genetic variation is most pronounced in the viral envelope gene. Phylogenetic analyses of diverse envelope sequences have shown that HIV-1 can be classified into multiple genetic clades or subtypes. Thus, ten genetic subtypes named A through J in group M (major) and a group of other subtypes (group O, outlying) have been identified. Amino acid sequence variation within a subtype generally ranges from 5 to 20%, and between subtypes from 25 to 35%. All genetic subtypes have been found in Central African countries, whereas subtype B predominates in Europe and the United States.

We isolated DNA from peripheral blood mononuclear cells of HIV seropositive individuals and AIDS patients of the St. László Hospital and applied HMA and genomic sequencing to classify the HIV-1 strains prevalent in Hungary.

HMA is a rapid method for the detection and estimation of genetic divergence between HIV strains on the basis of the observation that DNA heteroduplexes formed between related sequences have a reduced mobility in polyacrylamide gels proportional to their degree of divergence.

For genomic sequencing we amplified the V3 region of env using nested primers. The biotin labeled strand was bound on magnetic beads and sequenced using a fluorescein-labeled primer.

The results we got using HMA were in good agreement with the data obtained by genomic sequencing.

Our study shows that subtype B is the predominant HIV-1 clade at present in Hungary.

K. NAGY, B. KEMÉNY, A. HORVÁTH

Seroprevalence of Kaposi's sarcoma associated herpesvirus in HIV infected and healthy population of Hungary

AIDS and human retrovirus-laboratories, National Institute Dermato-Venereology, Budapest, Hungary

Based on our previous studies with KSHV-DNA amplification (Nagy K. et al., Cancer Control, 1997) we proposed, that KSHV might be present in the healthy population as latent, persistent infection.

In this report we analyzed the presence and prevalence of the KSHV antibodies in sera of 700 Hungarian individuals. 570 sera were from healthy subjects, 40 sera from asymptomatic HIV-1 infected persons under care of our institute, 40 sera from the HIV-negative sexual contacts of the previous group, 40 sera from STD patients, and 10 sera from classic KS patients.

Immunoreactivity of the sera were determined on KSHV-positive, inactivated, but not fixed BCP-1 cells (obtained from R. Weiss, with permission of P. Moore) by indirect immunofluorescence. All sera had also been screened for HIV-1 antibodies-by Elisa.

KSHV antibodies were detected in 80% of classic KS, and 25% of HIV-1 infected groups. Interestingly sera of HIV-negative contacts and those with STDs proved to be negative for KSHV antibodies. However among the 570 healthy individuals 14 sera (2.5%) showed strong, brilliant fluorescence proving the presence of KSHV antibodies. Titres were 1:100 – 1:2.000.

This study is the first survey for the prevalence of KSHV antibodies in the general population in our country. Here we provide experimental seroepidemiological data that KSHV infection – even if at low percentage – is present in the healthy Hungarian population. Its significance includes i) the possibility that the presence of KSHV antibodies may be one of the prediction progression markers of the subsequent development of KS in some of these individuals, ii) healthy blood donors may latently

have the transmissible KSHV, which may cause difficulties in controlling the security of blood transfusions.

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Antibodies to HHV-6 in HIV-infected subjects in Hungary

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The possible cofactors activating HIV and promoting AIDS have not been studied in Hungary yet. Among heterologous viruses known to play a role in these processes, human herpesvirus type 6 (HHV-6) might one of the most important, as data obtained world wide demonstrate. No data on HHV-6 prevalence and its possible role as a risk factor for AIDS have been revealed in our geographical region. Therefore, we studied prevalence and type of antibodies among HIV-infected patients, HIV-seronegative high risk subjects, few cases of other disorders, and controls. Serum samples were assayed by indirect immunofluorescence using JJHAN cells infected with HHV-6 U1102 and anti-human IgM and IgG, respectively. To verify positive cases, an ELISA-method is under development. Approx. two-third of control persons was found to carry low level IgG antibody in their sera, which suggest childhood infection. Among HIV seronegative high risk persons, prevalence of IgG was 100%: the level of IgG was moderate, but the majority of persons had high IgM titer. The persisting elevated IgM content indicates continuous reinfection and it might be due to promiscuous behaviour. Although all patients with HIV seropositivity showed presence of low level IgG, in their group the level of IgM was low. The latter might be explained by changing lifestyle after HIV serodiagnosis, consequently, preventing reinfection with heterologous viruses. Similar observations in alteration of seroepidemiology have been reported lately in Western countries. Our data suggest that, HHV-6 circulates and might play a role in AIDS development in Central Europe.

Supported partially by grants of SOTE, OTKA and NAB.

K. NAGY, B. KEMÉNY and A. HORVÁTH

Co-receptors, resistance and the promiscuous HIV

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During infection HIV-1 and HIV-2 bind to CD4 molecules and co-receptors localised on the cell membrane. The co-receptors determine the virus tropism, i.e. HIV strains with different tropism require different co-receptors.

CXCR4 (LESTR, fusin) is a member of the receptor family binding G-protein and serves as co-receptor for the syncytia inducing (SI), T-cell adapted HIV-1. *CCR5*, the other member of the β -chemokine receptors is the co-receptor for non-syncytia inducing (NSI), macrophage-tropic HIVs.

As the majority of the sexually transmitted HIV-1 isolates have the NSI phenotype and are macrophage-tropic, we studied the presence and the mutation of the gene coding for CCR5 receptor in long-term non-producer (LTNP) HIV-infected persons, their contacts as well as in healthy individuals, by using CCR5.759 clone. The 182 bp fragment of the intact CCR5 and the 150 bp deletion fragment of the CCR5 $\Delta 32$ were analysed by PCR.

The LTNP male patients have been infected since 8–12 years (mean 10 years), their CD4 counts are 118–485 (mean 317). We found 40 per cent of them heterozygote mutations of the CCR5 co-receptor. One of them has been infected since 12 years, with a CD4 count of 442. We also studied 2 female and 4 male HIV-negative patients, who have been sexual contacts of the LTNP patients since 3–6 years. In this group 33 per cent heterozygote mutants were detected, all males, as well as in some cases of healthy individuals. Homozygote mutation for the CCR5 gene has not been found. The high prevalence of heterozygote mutations of CCR5 among LTNPs supports findings that mutations in co-receptor gene provide a certain protection against HIV infection, and/or contribute to the reduced progression of AIDS. However the detection of promiscuous HIV-1 isolates changing their co-receptors makes the characterisation of selective resistance more difficult.

VIROLOGY

J. KÓNYA, GY. VERESS, K. SZARKA, A. JUHÁSZ, L. GERGELY

**A candidate cytotoxic T-lymphocyte epitope encoded
by human papillomavirus (HPV) type 16 E2
open reading frame**

Department of Microbiology, University Medical School of Debrecen, Hungary

Persistent infection with the oncogenic types of human papillomaviruses (HPV) is the major cause of the cervical cancer precursor lesions. Cellular immune responses are considered important in the elimination of HPV infection, but the targets are not well defined. The HPV E1 and E2 proteins form a replicative complex necessary for episomal genome maintenance. To investigate whether epitopes in the E1 or E2 proteins can serve as targets for cytotoxic T-lymphocyte (CTL)-mediated killing, we identified the peptides containing human leukocyte antigen (HLA)-A*0201 binding motif in the deduced amino acid sequences of the HPV type 16 E1 and E2 open reading frames. The binding affinity of the peptides was measured by HLA-A*0201 upregulation on T2 cells. Peptides with high binding affinity were tested for ability to elicit peptide-specific CTLs from healthy blood donors. We found one peptide from the E1 and one from the E2 protein sequence that were capable of eliciting peptide-specific CTLs. The E2 specific CTLs lysed an HPV16-transfected cervical carcinoma cell line, but not the untransfected HPV-negative parental cell line, indicating that the identified E2 epitope can be presented to CTLs in HPV-positive epithelial cells.

T. MAJOR, L. GERGELY, J. CZEGLÉDY

**Detection of human papillomavirus gene sequences
in laryngeal tumours and precancerous lesions
by polymerase chain reaction**

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Human papillomavirus gene sequences have been detected in a number of malignant and benign tumours. Non-oncogenic types 6 and 11 are etiological factors of benign mucosal tumours. Types 16 and 18 can be detected in malignancies most often but their role in the etiopathogenesis of cancers is still unclear. In our study we examined formalin-fixed and paraffin-embedded archive laryngeal tissues (paraffin blocks) containing squamous cell carcinomas (n=26), papillomas (n=8) and precancerous lesions (n=8) for the presence of human papillomavirus genes. As a control we also examined tissues harbouring laryngeal nodules (n=10) which represented the normal larynx in our study. After DNA preparation from the paraffin blocks we performed polymerase chain

reaction to detect the DNA of human papillomavirus types 6, 11, 16 and 18. The frequency of human papillomavirus gene sequences was higher in squamous cell carcinomas (11/26), papillomas (5/8) and precancerous lesions (3/8) in comparison with the control group (1/10). HPV 16 and 18 accumulated in carcinomas while HPV 6 and 11 were more frequent in papillomas. In precancerous lesions we detected the DNA of both oncogenic and non-oncogenic types. To verify the integrity of DNA we also amplified a sequence deriving from the cellular β -globin gene. Based on the 100% positivity for this gene we declare that the combination of our DNA preparation and polymerase chain reaction is a reliable method for detecting DNA sequences from formalin-fixed and paraffin-embedded tissues.

I. MEZEY, JIURU XIE, M. VARGA, E. FERENCZI, E. TÓTH, Á. FRANK-KISS
and GY. BERENCSI

Possible role of human parvovirus B19 in central nervous system diseases

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During the years 1990–96 more than 1400 sera from patients suffering from acute exanthematous disease and/or arthralgia were verified by the detection of the presence of human parvovirus B19 IgM antibodies by micro-ELISA. This was shown to be the first serologically proven parvovirus B19 epidemic in Hungary. Among the 362 patients with proven parvovirus infection there were seven persons having involvement of the central nervous system (CNS) as well. Altogether one man and 6 women – between 15 and 42 years of age – had meningitis or other signs such as dizziness, head-ache, vomitus and diplopia. During the above period overt circulation of any other viruses did not occur. Possible etiology of the following viruses known to cause diseases of the CNS were excluded by serological tests carried out on preserved samples, i.e. the role of rubella-, measles-, mumps-, tick-borne encephalitis-, LCM-, herpes simplex-, Epstein-Barr, and cytomegaloviruses could be excluded in the case of each patients. Virus isolation experiments gave negative results in the two patients investigated for enteroviruses. In the serum sample of one patient – submitted for parvovirus serology because of anaemia and thrombopenia, parvovirus DNS had been detected by nested PCR technique. Nevertheless a few days later meningitis developed. We believe that these patients were suffering in a possible “parainfectious disease” of the CNS caused by human parvovirus B19.

¹GY. SZŰCS, ¹M. ÚJ, ²I. MIHÁLY, ³J. DEÁK

Burden of rotavirus-associated hospitalizations in areas of Pécs, Budapest and Szeged

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Of viral gastroenteritis, rotaviruses are known to be the most important cause of acute gastroenteritis requiring hospitalization of infants and young children worldwide. Informations are not available on the burden and financial consequence of rotavirus-associated hospitalizations in Hungary. We provide epidemiological data based upon records of hospital admissions and laboratory reports of confirmed group A rotavirus infections of children ≤ 14 years old in three geographic regions of Hungary. Furthermore, these data were used to estimate the total number and cost of hospitalizations per year due to rotavirus infection. Between January 1993 and December 1996, 9182 hospitalizations for gastroenteritis occurred in the pediatric wards of hospitals in Baranya County, in the Szent László Hospital, Budapest, the Department of Pediatrics, Albert Szent-Györgyi University Medical School of Szeged, and the infectious disease ward of Csongrád County Hospital. Of the 9182 gastroenteritis cases, 1946 (21%) were associated with rotavirus infection. Most (90%) of the rotavirus detections were among children ≤ 4 years old. By extrapolation, an estimated 5000 rotavirus-related hospitalizations (18.6 / 1000 children ≤ 4 years old / year) occurred in Hungary during the study period. The estimated annual cost for these cases is \$5.04 million. Marked seasonality of rotavirus infections was observed with a peak of incidence from December to February. Rotaviruses with "long" RNA electropherotypes predominated each year but in 1995/1996 20% of electropherotypes in Budapest area were "short".

Effective surveillance is required for all children hospitalized for diarrhea as part of a possible rotavirus immunization program in the future in Hungary.

¹I. MIHÁLY, ¹A. LUKÁCS, ²L. TELEGDY, ³E. IBRÁNYI

Prevalence of hepatitis C antibody in hospital personnel

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The health-care workers are known to be at risk of occupational transmission of blood-borne viruses. The goal of the investigation was to determine the prevalence of hepatitis C virus (HCV) antibody and the occupational risk of HCV transmission among personnel at the Central Hospital for Infectious Diseases, Budapest; Hungary. Serum samples of 500 health-care workers were tested for antibody to HCV with second and third generation ELISA-s and anti-HCV positive sample: were confirmed with Western

Blot Line EIA. Amplicor HCV-RNA test were done in the HCV seropositive workers. A total of 12 (2.4%) of the health-care workers were confirmed to be anti-HCV positive. The prevalence of anti-HCV increased with advancing age: zero under 20 yr age group, 0.9% in 21–30 yr age group, 3.6% in 31–40 yr age group, 3.1% in 41–50 yr age group and 4.0% in above 50 yr age group. Seven of the twelve HCV antibody positive workers had HCV-RNA in their sera intermittently (58.3%). We found anti-HCV positive hospital worker in 10 out of 17 departments. The prevalence of hepatitis C antibody was 7.1–1.9% among the personnel of internal departments, pathology, intensive care unit, surgery and pediatric departments. No anti-HCV positive health-care worker was found in laboratories. Eight of the nurses, two of the physicians, one of the sanitary personnel and one pathological technician were seropositive for HCV. Two nurses developed chronic C hepatitis after a needlestick accident.

Conclusions: 1. The hospital personnel is at risk for HCV infection. 2. The occupational risk of HCV infection increases with age but the risk is considerable lower than that of hepatitis B infection. 3. The occupational risk is highest among the workers of the chronic internal department, pathology and intensive care unit. 4. The nurses are at higher risk of HCV infection than the physicians. 5. The needlestick injury is associated with an increased risk for acquiring HCV infection.

É. ÁDÁM, I. NÁSZ, A. LENGYEL, O. DOBAY

Studies on intertype specific epitopes of adenovirus hexons using antibodies directed against synthetic peptides

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The use of monoclonal antibodies against hexons of different adenovirus serotypes resulted in the recognition that the antigenic structure of the capsomer is more complicated than it was reported earlier. On the base of these experiments we have shown that besides the genus and type specific epitopes a great variety of intertype specific epitopes could be found on hexons in various combinations.

To study the intertype specific epitope polypeptides were synthesized and used in the experiments. Before the synthesis of polypeptides, the possible regions with epitope properties were predicted using methods based on statistic analysis of β -barrel structures, hydrophilic, flexible and surface exposed protein regions. Six possible amino acid sequences were predicted and then synthesized. To induce immune response against these low molecular weight polypeptides, bioconjugates were prepared with poly(L-lysine) backbone. To produce antibodies Balb/c mice were immunized i.p. After the six times repeated injection, the developed ascitic fluids were collected and used in ELISA experiments to study the epitopes of different purified adenovirus hexons.

It was found that the developed bioconjugate were able to induce antibody production in mice and anti-peptide antibodies were able to bind to the adenovirus hexons recognizing antigenic sites on them. All the six anti-peptide antibodies reacted with the hexons of subgenus C, and in different combinations with the other hexon types studied.

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Prevalence of a new flavivirus hepatitis in Hungary

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Sera of blood donors with elevated transaminases and sera of patients suffering from non-A-E hepatitis were found to be reactive to reagents prepared from the yellow fever flavivirus vaccine strain. Using GBV-B primers PCR products were obtained. None of the other GBV (GBV-A and GBV-C) viruses were found in seropositive samples. The sequences of the PCR products were different from all published flavivirus-like hepatitis viruses. The same amplicon-sequences were identified from a healthy blood donor and non-A-E hepatitis patients.

A. HAJNAL

Evaluation of hepatitis virus serological studies in blood bank service and patient care

National Center of Blood Bank, Budapest, Hungary

Similarly to other Blood Banks, the National Center of Blood Bank is involved in virus serological investigations in both blood bank and patient care service. The present work focuses on analysis of ELISA data of HBs antigen and anti-HCV in 1995–96.

1. During this period the number of claims for anti-HCV determinations in general patient's care increased significantly. It is approved that compared to blood donors, the positivity among patients for anti-HCV and HBs Ag is considerably higher.

2. The exclusion of serologically (ELISA) positive donors from blood donation is performed only after further confirmation (verification). However, the release of a record about serological positivity in patients is performed based on ELISA data alone.

This presentation intends to emphasize the differences between blood donors and patients concerning the subsequent evaluation of virus serological results.

¹L. LŐRINCZI, ²GY. SZEKERES, ²P. NÉMETH

Detection of HBx antigen in liver biopsy samples

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The epidemiological data suggest that chronic infection with hepatitis-B virus (HBV) is associated with high risk for the development of hepatocellular carcinoma (HCC). The X gene product of the HBV genome (HBxAg) functions as a transactivating element and it is considered as an essential factor for establishing chronic infection as well as mediating carcinogenesis. HBx expression in the liver during chronic HBV infection may be an important prognostic marker for the development of HCC.

Sections from formalin fixed and paraffin embedded liver biopsy samples were examined immunohistochemically for HBx, HBs and HBc antigens.

These antigens were studied in cases of chronic hepatitis, cirrhosis and HCC.

Our results indicate that a higher rate of positivity was found for HBxAg than for HBs and HBc antigens.

¹I. MIHÁLY, ¹A. LUKÁCS, ²L. TELEGDİ, ³E. IBRÁNYI, ²T. SZILÁGYI
and ⁴N. KESZEI

Etiology of acute hepatitis in health care workers of "St László" Hospital, Budapest

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A total of 215 sera of 55 hospital workers suffering in acute hepatitis were tested for hepatitis A (HAV), hepatitis B (BV), hepatitis C (HCV), hepatitis D (HDV), hepatitis E (HEV), Epstein-Barr virus (EBV) and cytomegalovirus (CMV) from 1985 till 1997. Sera from the preceding time of hepatitis from 32 workers (58%) and one or more sera following the illness from 37 workers (67%) were examined. The viral etiology was determined in 38 patients (69%). Sixteen hepatitis cases were connected with HBV (29%), 6, 6, 6 hepatitis cases were caused by HAV, HCV, HEV, respectively (11, 11, 11%). Only three of six "A" hepatitis patients had anti-HAV IgM antibody during the acute hepatitis phase, in the order three "A" hepatitis cases the etiology was determined by seroconversion to HAV during or soon after the time of clinical symptoms. Six workers had anti-HCV IgG antibody in the time or soon after the acute hepatitis. One of the six HCV seropositive workers seroconverted to hepatitis C virus during the illness following a needle accident. Six persons had antibody to HEV (11%), three of them seroconverted to HEV during the clinical disease. Two of the workers had acute hepatitis caused by cytomegalovirus (4%). In two of the patients seropositivity was found against

more than one hepatitis virus. Two of the workers had toxic hepatitis and in 15 of the workers the etiology remained unknown (27%).

Conclusions: 1. Hepatitis B virus plays a leading etiological role in acute hepatitis among health care workers also in the vaccination era. 2. The frequency of the "A", "C", "E" hepatitis cases was the same among the hospital personnel. 3. Etiology can be determined exactly only by several different cautious examinations. 4. It is recommended to draw blood regularly from hospital personnel and to store the samples.

¹I. MIHÁLY, ¹A. LUKÁCS, ²E. IBRÁNYI, ³L. TELEGDI, ⁴E. NAGY

**The changes of etiology of the viral hepatitises from 1991
to 1997 in patients admitted to the St László central hospital
for infectious diseases**

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The authors tested 4424 patients with acute hepatitis by serological methods for HAV, HBV, EBV and CMV infection between 1991 and 1995. Patients were divided into 4 groups: 3823 patients in the sporadic group, 252 patients in the post-transfusion group, 227 in the post-inoculation group and 122 in the health workers' group. In the sporadic group, the etiology could be determined in 43% of the patients, 22.8% having been due to HAV, 13.1% to HBV, 3.3% to HCV, 2.5% to EBV and 1.3% to CMV infection. In both the post transfusion and post-inoculation group HCV infection was the leading etiology. However, there was a significant difference concerning the frequency of HCV positivity between the two groups (42.3% in the post transfusion group vs 13.8% in the post-inoculation group: $p < 0.001$). This difference points to the utmost importance of blood transfusion even as compared to the inoculation route in transmitting to HCV infection. In 1993, the year of introduction of screening of donors for HCV antibody in Hungary, the number and percentage of post transfusion hepatitis in this study dropped abruptly and remained at low level in the following years (8.3% in 1991, 8.8% in 1992, 3.7% in 1993, 4.5% in 1994, 3.0% in 1995). The percentage of HBV infection was 11 (28 pts) in the post-transfusion group and 13.1 (30 pts) in the post-inoculation group. In the health care workers' group the etiological agents were as follows: HBV in 21.3% (26 pts), HCV in 6.5% (8 pts), HAV in 4.9% (6 pts), CMV in 2.5% (3 pts), EBV in 1.6% (2 pts). During the 5 years delta coinfection could be detected in 8 cases, among them 2 in the health care workers' group. Recombinant F-3EV antibody test was performed in 63 sporadic and in 14 post-inoculation hepatitises which according to the previous tests were non-A, non-B non-C cases and in 46 patients in the health care workers' group. Among them HEV IgG reactive were 15 cases (23.8%) in sporadic group, 3 cases (21.4%) in the post-inoculation group and 18 (31.8%) in the health care workers' group; as confirmed in repeated tests.

CS. JENEY, O. DOBAY, É. ÁDÁM, I. NÁSZ

Elimination of agarose contamination from DNA samples

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The preparation of DNA from agarose gels is a central question of molecular genetics. Numerous methods and many modifications have been worked out in the last years which indicates the importance of this problem. These methods basically belong to three categories: electrophoretic, affinity and physical methods. Nowadays mainly the last two kinds of methods become conspicuous, since the electrophoretic procedures are much more time consuming than the others. In case of affinity methods there are problems with the reproducibility, this is the reason of widespread use of commercial kits, since the parameters of matrix preparation can be controlled just under industrial circumstances. These methods are quite expensive and despite of the controlled production they still cause reproducibility problems. The physical methods are cheap and dependable. They are based on the breakdown of agarose gel by pressure or centrifugal force followed by squeezing out the DNA-containing buffer from it through a filter. However this DNA-solution is never clear enough, it contains buffer components and agarose contamination, this is why spectrophotometry is not applicable for measuring the DNA-content.

Getting over these difficulties we elaborated a method in order to remove the agarose contamination from DNA samples quickly, effectively and reproducibly what makes possible the quantification of DNA sample by spectrophotometry. In this method we use a so called "spincolumn" gelfiltration after the physical extraction of DNA solution. Using Sephacryl S-400 HR gelfiltration matrix a one-minute centrifugation is enough to recover the total volume. There is another advantage of this method that it results in buffer change streamlining the preparation of the following enzymatic steps. The whole preparation time of the DNA sample takes about 10 minutes and the reproducibility is very good. It can serve as a useful alternative of affinity methods, since it is more reproducible, more reliable, much quicker and cheaper than most of the affinity methods.

A. SZENDRŐI, M. EL-SAGEYER, I. MEZEY, GY. BERENCSEI

Differences in the sequences of the 5'-untranslated regions of field isolates of echovirus 11 and their effect on the secondary structure of the RNAs

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An echovirus 11 epidemic occurred in 1989 in Hungary, which caused haemorrhagic hepatitis of lethal outcome in the case of 15 newborn babies. The molecular comparison of the prototype echovirus 11 with the pathogenic virus isolates was the aim of this study. The virus isolates were shown to be the 'prime' strains of echovirus 11.

The first 100 cases of the 5'-untranslated region has the main function in RNA replication. The nucleotides up to the last initiation codon possess different functions in the initiation and regulation of translation. The central 435 nucleotides of the untranslated region have been serially amplified using RT-PCR (nucleotides 163 to 603). The amplimeres have been sequenced by Thermo Sequenase (Amersham) cycle sequencing kit with the help of a fluorescent primer located at the 168th nucleotide.

After the sequencing and aligning of 12 field isolates, the loops No. IV to VII. (D-G) of IRES region were found to be very similar to each other. Nevertheless, significant differences were observed in comparison to the prototype strain(s) and similar regions screened by the 'blast' programme. The closest sequence homology was found to the genome of swine vesicular disease enterovirus (D004s5-svdv). Cluster analysis of the available sequences grouped the analysed region of echovirus 11' RNA together with svdv and echoviruses 7 (L76400) and 9 (X92886).

The difference in the sequences suggest that the isolates from the Hungarian epidemic in 1989 might be the result of one or several recombination events between echovirus 11 and one of the members of the above cluster probably affecting the D and or G loops of the region.

BACTERIOLOGY

L. EMÓDY, F. KOVÁCS, M. KERÉNYI

Biological properties and clinical significance of *GVVPQ* fimbriae

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GVVPQ fimbriae, representing a new class of bacterial surface appendages, were described on *Salmonella enteritidis* in 1991. One year later their presence was detected also on fecal *Escherichia coli* isolates from protracted infantile diarrhoea. The name – *GVVPQ* – represents the amino acid sequence of the N-terminal region of the fimbrin subunit. Basing on this N-terminal sequence, another surface structure – originally described on bovine mastitis *E. coli* isolates as "curli" – proved to be identical to *GVVPQ* fimbriae. A striking characteristic of these structures is that they surround the bacterial cells like a plait of hair. It is important to note that they are not expressed on culture media conventionally used for diagnostic bacteriology. On the other hand, when a proper medium is applied (T-medium), there is a rich expression – unlike other fimbriae – also at room temperature. Fimbriate bacterial cells show a strong tendency to autoaggregate.

Comparative studies on the original *S. enteritidis* isolate and its *GVVPQ* fimbria negative mutant revealed that these fimbriae play an important role in interaction with

tissue matrix proteins (fibronectin, various types of collagen, and laminin), and promote adhesion to and penetration of epithelial cells.

Our present knowledge does not permit to draw firm conclusions on the pathogenetic role of *GVVPQ* fimbriae. We could, however, show by immunohistochemistry the expression of *GVVPQ* fimbriae in the organs of a person died in *S. enteritidis* sepsis. Both individual bacteria and large aggregates could be observed in the tissues. The ability of these fimbriae to promote cell adherence and penetration, and binding to matrix components of the host may contribute to bacterial colonization, cell penetration, and tissue invasion. Bacterial cells included in large tight aggregates may resist to adverse pH values and digestive enzymes, to the antibacterial effect of antibodies, complement and antimicrobial drugs, and also to the phagocytic process. Through all these effects *GVVPQ* fimbriae may potentiate the *in vivo* resistance and invasiveness of the pathogen, and in this way the development of systemic infections.

GVVPQ fimbriae may also serve as an additional character in biotyping of the isolates for diagnostic or epidemiological purposes.

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**Relationship between fimbria production, matrix protein
binding and organotropism in uropathogenic
*Escherichia coli***

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The frequent occurrence of S-fimbriated bacteria in systemic infections caused by *Escherichia coli* is well known. It is presumed that these fimbriae are involved in the development of systemic infection rather than mucosal colonization at the site of entry. Examinations on frozen kidney sections have revealed the specific binding of purified S-fimbriae or S-fimbriated bacteria to the epithelial cells of the glomeruli, tubuli, collecting ducts or to the vascular endothelium. As the above experiments were performed by adding fimbriae or bacteria directly to the target tissues, it remained unanswered whether this tissue specificity exists in the case of septic manifestations when bacteria have to reach the organs through the circulation. Behaviour of an uropathogenic *Escherichia coli* strain producing S-fimbriae and its isogenic non-fimbriated derivative was compared in intravenous and intravesical mouse models to elucidate this question. Parallely performed matrix protein binding assays confirmed that S-fimbriae interacted with immobilized fibronectin, type I collagen and laminin.

The experiments in mice revealed that elimination of microbes injected into the circulation was much faster in the case of the fimbria negative derivative. Fimbriated bacteria possessed a higher degree of virulence measured by the lethal doses. They

showed obvious affinity not only to the kidney but also to the eye. This organotropism was proved not only by the results of quantitative bacteriological examinations but also by macroscopic and microscopic investigations of the organs.

Three weeks after intravesical infection the non-fimbriated derivative still persisted in the bladders of more than 50% of mice but it could not be detected in any of the kidneys. On the contrary all animals infected with S-fimbriated bacteria revealed positive bladder cultures 21 days after inoculation, and more than 90% of mice presented infection of the kidney. This latter result suggests that S-fimbriae may play a role in the process of ascension of the infection. Further investigations are, however, needed to clear up the mechanisms how S-fimbriae help the progression of infection at this step in the presence of soluble inhibitory compounds for fimbrial binding.

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^{1,2}V. TÓTH, ¹M. KERÉNYI, ¹E. PÁTRI, ³T. TOMCSÁNYI, ¹L. EMŐDY

Investigations on *Proteus penneri* virulence by in vitro and in vivo model systems

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Proteus penneri, earlier known as indol negative *Proteus vulgaris* or "*Proteus vulgaris* biogroup 1" has been implicated as a causative agent of urinary tract and wound infections, mostly of nosocomial origin. It has also been isolated from the faeces of healthy and diarrhoic individuals. Its causative role in enteric manifestations has, however, not been confirmed.

In our earlier studies we investigated the virulence properties of a *Proteus penneri* strain isolated in our diagnostic laboratory from a dermatology patient with wound infection. The aim of the present study was to evaluate the virulence of *P. penneri* on a broader scale, that is including a set of isolates representing various biotypes, geographical areas and clinical manifestations. The thirteen strains exhibited different proticin types, somatic antigen characters, plasmid profiles and RAPD-PCR patterns. Nine strains produced a diffusible hemolysin belonging to the RTX toxin family, and the remaining four isolates produced a cell associated hemolysin only. Beside these original isolates spontaneous and transposon mutants with hemolysin negative character were also included in the virulence studies.

The virulence of the strains showed a close correlation with the RTX-hemolytic capacity in both the in vitro and in vivo assays. Culture supernates of all the nine strains producing this type of toxin elicited a strong cytotoxic effect on all the three cell lines tested (HeLa, Hep-2, Vero). It is remarkable that the remaining four strains with cell-bound hemolysin only elicited a toxic effect in a direct cytotoxicity assay, when bacteria

were co-incubated with the eukaryotic cells. No such effect could be observed with the non-hemolytic mutants.

In the *in vivo* systems mice were infected intraperitoneally, intravenously or intranasally. The virulence of the strains in these models was also in close correlation with the capacity to produce the *RTX* toxin. The pivotal role of this hemolysin is supported by the observation that the degree of virulence in the animal assays and the amount of hemolysin produced *in vitro* showed a close correlation. Further, the development of a rapid hemorrhagic lung edema in intranasally infected mice strongly resembles the effect of other enteric bacteria producing *RTX* type toxins. It is suggested that the local toxic effect exerted by this hemolysin may play a role in the pathogenesis of urinary and wound infections caused by *P. penneri*.

This work was supported by the COPERNICUS No. CIPA CT 94-0230 and OTKA No. 016193 grants.

M. HERPAY, É. CZIRÓK, I. GADÓ and H. MILCH

How in Hungary we detect verocytotoxin producing *Escherichia coli* infection

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The objective of this study was to find tests suitable for proving the etiological role of verocytotoxin producing *E. coli* (VTEC). Specimens were screened by using sorbitol MacConkey (SMAC), Cefixim and tellurit (CT) SMAC and Entero-haemolysin (Ehly) agar, and tested for serotypes, phage types and VT (Verotox-F, VTEC Screen, Premier EHEC, DNA probes, PCR). Altogether 4532 faeces for presence of *E. coli* O157 and 545 *E. coli* strains, 526 mixed cultures, 49 faeces for VT were examined. Forty *E. coli* O157 were isolated. Eight, 14 and 31 out of Canadian phage types were observed among our VT positive O157 isolates. Twenty-one VTEC were detected: O26:H11 (4), O18ab:H- (2), O39:H48 (1), O76:HNT (2), O96:HNT (1), O157:H7 (3), O157:H33 (1), O157:HNT (3), O157:H- (4). Thirteen strains were VT1, two VT2 and three VT1+VT2 producers. VT positivity was verified only by DNA probes in 3 strains. Free VT were detected in 12 faeces. Seven mixed cultures and 4 isolates of them were VT positive, too. CT-SMAC proved to be selective both for O157 and for non-O157 VTEC. VT productivity of mixed culture is detectable with latex agglutination by our method. Successful VT diagnosis can be expected from different kinds of simultaneously done probes.

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**A novel 60 KDa exoprotein encoded by large plasmid
of enterohaemorrhagic *Escherichia coli* O157:H7**

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Analysing the large plasmid pO157-encoded exoproteins of enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 strain 7785 a presumably new, 60 kDa protein was detected. Exoproteins were precipitated from broth cultures by using trichloroacetic acid. The precipitate contains four species of proteins with molecular mass of 110-, 92-, 82- and 60 kDa analysed by SDS-PAGE. None of these proteins could be detected in a pO157-minus derivative strain 2-45 either by coomassie brilliant blue staining or by immunoblot (Western blot) analysis using pO157-specific immunoserum. The protein of 82 kDa first published by us could be the newly described catalase-peroxidase (katP) protein of 81.8 kDa. Protein of 110 kDa is the EHEC-haemolysin. Several pO157-specific exoproteins with the same and similar molecular weight were detected by western blot analysis in an *E. coli* O96:HNT strain isolated in Hungary from a patient suffering from haemorrhagic colitis. In a PCR reaction using pO157-specific primers DNA fragment of expected size was amplified. The strain harbours a large and two small plasmids.

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**Characterization of the plasmid pTC of the enterotoxigenic
Escherichia coli (ETEC) 2173**

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The *E. coli* O147:H+:F18ac strain 2173 has been isolated from a case of porcine postweaning diarrhoea, which is usually caused by enterotoxigenic *E. coli* (ETEC) strains producing heat stable toxins. STa and STb. Earlier studies of Olasz et al. indicated that the STb production is directed by a gene that is located on a relatively large (60–80 kb) plasmid of *E. coli* 2173, which is also responsible for tetracycline resistance (pTC). Here we report about the results achieved so far in characterization of this plasmid.

Conjugation abilities of pTC was tested to reveal whether the plasmid can be transmitted through conjugation from conjugative (*E. coli* S17-1) or from non-conjugative (*E. coli* TG1) bacterial strains to recipients from which the pTC had been removed (strains NBI). Plasmid pTC was able to transfer from both S17-1 and TG1.

Localization and typing of the origin of replication was attempted because it is regarded as part of the important informations to characterize Ec 2173 and the groups of ETEC represented by that strain. So far we have been successful to produce a deletion

mutant of pTC that carries the origin of replication and supply data for constructing the physical map.

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***Salmonella penarth* in Hungary, characterization
of the isolated strains**

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Officer Service of County Csongrád, Szeged, Hungary

Salmonella penarth, which was isolated first in South-Wales in 1952, occurred rarely since that time, it appeared first in Hungary in 1994 and in 1995 it caused family outbreaks in counties Győr-Moson-Sopron and Baranya and hospital epidemic in county Csongrád. The spread of the epidemic could be traced by the help of the phage-typing method which was elaborated by us by the adaptation of 9 phages (*S. enteritidis*, *S. typhimurium*, *S. panama* and *S. sonnei*) to *S. penarth* strains. A total of 105 strains were examined, the strains isolated in county Győr-Moson-Sopron belonged to phage-types 118 and 111, the strains derived from county Baranya were of phage-types 461 and 118. The strains isolated in county Csongrád belonged to phage-type 111 in 92.7%. Strains, which showed different phage-types isolated repeatedly from the same persons differed also in their antibiotic sensitivity, and/or bacteriocin-production and plasmid profile. The examined strains were sensitive to antibiotics in 22.9%, multiple resistant in 72.4%. A plasmid of size 120 Mdal was carried by 72.3% of the strains, this plasmid was significantly more frequent among the strains isolated from patients than from carriers. Conjugation experiments proved that resistance determinants were carried by these plasmids. Some other big plasmids were also found, but small plasmids (<10 Mdal) were not present. Changes of the phage-types of the recipient were developed by conjugation. There was no connection between the presence of 120 Mdal plasmid and the effect observed in Serény-test in guinea-pig. The strains carrying the 120 Mdal plasmid in situ hybridized with the ipaH invasivity-probe of *S. flexneri* origin labelled with ³²P in 89.3%, the strains which did not contain this plasmid did not hybridize.

E. NAGY, E. FODOR, Z. PRÁGAI, I. VERÉB

**Detection of different resistance mechanisms against
beta-lactam antibiotics by molecular biological methods**

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Szeged, Hungary*

Resistance to beta-lactam antibiotics can develop through different resistance mechanisms such as changes of PBPs, decreased permeability of membranes and the production of beta-lactamases with different substrate spectrum. The detection of the presence of these resistance mechanisms by using the classical in vitro tests is sometimes difficult. Molecular biological methods may help to detect the presence and the spread of these resistance mechanisms among clinical isolates. The presence of the *mecA* gene is responsible for the multiresistance to beta-lactam antibiotics among staphylococci. The expression of the gene is influenced by many factors. PCR method was used to detect the *mecA* gene among clinical isolates of *Staphylococcus aureus* (44) and coagulase-negative staphylococci (CNS) (45) and the MICs of oxacillin were also compared. In the case of two CNS blood isolates we could detect the *mecA* gene without elevated MICs against oxacillin. One *S. aureus* and one CNS isolate showed high MICs of oxacillin, while the *mecA* gene was not detectable. All other isolates showed concordant results.

Extended spectrum beta-lactamase (ESBL) was found in 8.8% of the *Klebsiella pneumoniae* strains derived from 6 centres of Hungary and the PCR-SSCP method confirmed the parallel presence of SHV2 and SHVS among the Hungarian isolates. *Serratia marcescens* strains isolated from a nosocomial outbreak revealed the presence of the Class I chromosomally mediated cephalosporinase and the plasmid mediated SHV2 type ESBL suggesting the in vivo acquiring the gene by plasmid transfer during the outbreak.

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¹E. WESZELOVSZKY, ³J. SZÖLLŐSI, ⁴I. TÓTH-KÁSA, ⁴M. FÁRK, ⁵S. NAGYMIHÁLY
and ⁶T. NYÁRI

**Prevalence of STD pathogens in an HIV sentinel
group of females**

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Highway "sex-workers" (catering to truck drivers and others travelling between Western Europe and the Balkan states or the Middle East) and town "sex-workers" were screened for STDs.

Neisseria gonorrhoeae, *Chlamydia trachomatis* and HPV were identified by nucleic acid hybridization. HSV, bacterial pathogens, *Mycoplasma hominis* and *Trichomonas vaginalis* were cultivated. Anti-HIV-1/-2, anti-HSV-1/-2, anti-CMV, different hepatitis markers and anti-*Treponema pallidum* titres were determined in serum samples. Drug metabolites were determined in urine samples.

100 highway "sex-workers" were screened. Only 44 per cent of the cases were negative. One pathogen was detected in 26 per cent, two pathogens in 11 per cent and three or more pathogens in 19 per cent of the samples. Of 455 town "sex-workers", 14.7 per cent were positive, and only in 3 cases was a double infection detected.

With the introduction of systematic screening and treatment, the spreading of STDs may be recognized and prevented. The highway sex-workers are in greater danger and pose a greater danger to their clients than do the city sex workers. It is essential to call attention to the use of condom contraceptives.

These investigations were sponsored by the Hungarian AIDS Foundation.

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Characterization of *Actinobacillus pleuropneumoniae* biotype 2 strains isolated from swine

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Although *Actinobacillus pleuropneumoniae* biotype 2 strains as *Pasteurella haemolytica*-like bacteria were described 18 years ago, the knowledge on the occurrence and the pathology of this bacterium is limited.

During the last 8 years 77 *A. pleuropneumoniae* biotype 2 strains were isolated from 63 swine carcasses sent for post mortem examination from 33 herds. Haemorrhagic, fibrinous-necrotic pneumonia with abundant exudation and fibrinous pleurisy were observed in the apical and the diaphragmatic lobes uni- or bilaterally. The lung lesions cannot be distinguished from that of caused by *A. pleuropneumoniae* biotype 1 strains but the latter biotype is highly contagious, while *A. pleuropneumoniae* biotype 2 strains cause only sporadic diseases. Environmental factors and other infections can predispose the pigs to disease caused by *A. pleuropneumoniae* biotype 2 strains.

Sixty-one *A. pleuropneumoniae* biotype 2 strains were cultured from lung lesions, 7 from spleen, 5 from kidney, 2 from liver and one from endocardium and a fetus. The isolates were biochemically uniform. Using the indirect haemagglutination test 40 strains belonged to the proposed serotype 1 and 35 to serotype 2 while 2 strains could not be assigned into either serotype. The latter strains had shared antigens with the type-strain of serotype 1 in the double gel diffusion test but the type-specific antigen of the type-strain was missing in the untypable *A. pleuropneumoniae* strains.

¹B. SZABÓ, ¹C. MISZTI, ¹L. MAJOROS, ²Z. NÁBRÁDI, ³SZ. GOMBA

**Isolation, characterisation and studies on virulence
and antibiotic sensitivity of *Rhodococcus equi***

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Rhodococcus equi is a well-established pathogen in foal pneumonia and is increasingly recognized as a pathogen in immunocompromised humans. We have isolated a Gram-positive coccobacillus from 8 blood samples and lung tissues of a renal transplant patient. On the basis of 21 biochemical reactions, the characteristic morphological cycle (coccus-rod-coccus) and the positive CAMP test *Rhodococcus equi* diagnosis was established. The high virulence of *R. equi* seems to be associated with the presence of a 80 kD plasmid and the adherence to HeLa cells. Our isolates proved to be less reactive in the latter test. Histological studies of 6 lung biopsy specimens revealed numerous microabscesses with aggregates of polymorphonuclear leukocytes surrounded by abundant foamy macrophages. Tissue Gram's strains demonstrated numerous extracellular and few intracellular Gram-positive coccobacilli characteristic of *R. equi*. A correlation can be hypothesised between the relative low level in vitro virulence of our isolates and the moderate in vivo intracellular appearance of *R. equi*.

Our isolates proved to be sensitive to majority of antibacterial drugs except some cephalosporins, macrolides and trimethoprim. The amoxicillin-clavulanate therapy was effective.

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Naturally occurring bacterial endosymbionts

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Small, "free-living" amoebae such as *Naegleria* and *Acanthamoeba* were found to contain bacterial endosymbionts. The role of such endosymbionts in the pathogenesis is unknown, though they have been implicated in the development of amoebic keratitis. Certain studies have speculated on the possibility that endosymbionts act as potential virulence factors. We organized systemic cooperative study for the isolation, identification and possible pathogenicity of the fauna of the "free-living" amoebae in Hungary, with special reference to the sites of human recreation, such as natural waters, hot springs and swimming pools. Comparing the restriction fragment length profiles (RFLP) of the isolates by the use of restriction endonuclease digestion of mitochondrial DNA (mtDNA), we have identified intracellular bacteria in an axenically grown *Acanthamoeba* sp. isolated from the basin of a natural hot water spring. Electron

microscopy revealed evidence for multiplication of the rod-shape bacteria within the amoebic cytoplasm. The identification and characterization of that endosymbiont as well as its possible role in any pathogenic process is the subject of further examinations.

¹I. KUSTOS, ¹B. KOCSIS, ²F. KILÁR

Determination of protein profile and outer membrane proteins of bacteria by capillary electrophoresis

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Determination of bacterial protein profile is a suitable method for identification and comparative studies of bacteria. This means the characterization of total protein content found after lysis of bacterial cells. The protein profile has been determined in different strains of *Morganella*, *Bartonella*, *Listeria*, *Pseudomonas*, *Agrobacterium*, *Zymomonas* genera. According to data in literature these investigations were performed only by SDS-PAGE.

Our experimental purpose was the determination of protein profile by capillary electrophoresis (CE). Dynamic sieving CE was applied, with a hydrophil polymer as a sieving agent, and this enables the separation of proteins on the basis of their molecular weight. Advantages of CE to conventional slab gel electrophoresis are the fast separation, on-line detection, and small amount of sample.

Different strains of *Enterobacteriaceae* family were investigated: *Proteus penneri* 357, *Escherichia coli* 575, *Shigella sonnei* 4350, *Salmonella minnesota* R595. There were significant differences in the number, height, and migration time of the peaks in the different strains. The patterns were characteristic for the bacteria investigated, and they were reproducible. The molecular weight of the characteristic peaks in the patterns were determined (*P. penneri* 357: 14, 30, 41, 44, 73 kD; *E. coli* 575: 16, 24, 31, 42, 27, 55 kD; *S. sonnei* 4350: 16, 31, 42, 57 kD; *S. minnesota* R595: 9, 16, 24, 42, 45, 54 kD). Results were obtained in 20 minutes.

In the second part of our experiments the outer membrane proteins (OMP) of the same strains were examined. There was good correlation between the results of CE and SDS-PAGE, but with CE the separation was completed in 15 minutes, and the resolution was better. The molecular weight of major OMP-s was determined by the help of an internal standard (benzoic acid) and a calibration kit (Pharmacia) (*P. penneri* 357: 27, 41 kD; *E. coli* 575: 20, 22, 30, 41; 42 kD; *S. sonnei* 4350: 16, 18, 34, 36 kD; *S. minnesota* R595: 16, 28, 39 kD).

Based on their results the authors presume that CE is a suitable method for determination of protein profile and OMP-s in microbiological diagnostics and this technique has a lot of advantages compared to the methods used earlier.

B. P. BOZSIK

Lyme borreliosis seronegativa

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Lyme borreliosis is a diagnosis based on consultation, where case history is highly important, the data obtained during clinical examinations and treatment are inevitable, and verification in the laboratory is of critical importance. Diagnosing Lyme borreliosis is made difficult by diverse clinical manifestations, the number of diseases and syndromes of unknown origin that can be associated with it, the low number of specific symptoms and the fact that specific symptoms are often missing. There are no clear rules of how the clinical picture develops, and there can be two or more other diseases during the chronic illness. These may be treated, thereby modifying differently the whole picture of Lyme borreliosis.

Therefore, it is through a complex process that we have to decide whether we usually diagnose Lyme borreliosis less or more frequently than its natural prevalence. In my opinion, it is impossible to overdiagnose them, as the cumulative prevalence of the disease in Hungary could reach as much as one million.

Seropositivity itself, however, cannot be the exclusive basis for verifying Lyme borreliosis, because

Seronegative Lyme borreliosis is part of the natural process of the disease, due to its special immunological properties. Diagnostic difficulties may be caused by seronegativity that can appear any time, not only at the beginning of the disease.

It has been widely known with diseases caused by Spirochetes that early antibiotic treatment prevents antibody production for a lifetime and causes a seronegative picture.

Because of failing to cultivate the bacteria and the low number of Spirochetes, it was necessary to develop a new method for the laboratory to verify seronegative Lyme borreliosis. I have been running these examinations for twelve years now, my results can be summarized as follows:

I have video documentary about the causative agent being present in the blood of seronegative patients. I can show the different forms of *Borrelia burgdorferi*, their motion, duplication, shedding, and forms after their shedding.

As a negative control, there are different cells of blood with hardened membrane by a reagent, not allowing them to produce artificial products, and the so-called myeloid figures.

As a positive control, there are immunocytological results with monoclonal antibodies against OspA and flagellin kindly gifted by prof. Barbour and results of different kind serological kits.

By interpreting the microanatomical changes of *Borrelia burgdorferi* we have been enabled to give new answers to the questions asked during the pathogenesis and clinical course of Lyme borreliosis, these answers then could guide the diagnosis and the therapy.

B. P. BOZSIK

Lyme borreliosis seropositiva

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The pathogenesis of many diseases and syndromes of unknown origin was clarified with the discovery of Lyme borreliosis and its causative agent (*Borrelia burgdorferi*, 1982). The discovery has also made it possible to cure these diseases more effectively.

In the beginning serodiagnosis was done in the classical way. However, as the clinical picture was investigated, unusual events became known:

IgM antibodies could be detected for years, in some cases exclusively, without the presence of IgG-type antibodies.

There was major discrepancy between the seriousness of the clinical picture and the laboratory findings: negative serological result was joined with typical symptoms, and a positive result was found although the patient was healthy.

Laboratory diagnostics was further hampered by the fact that there was a high level of inconsistency between the results in different laboratories, even after identifying and applying different substrains of *Borrelia burgdorferi*.

As a result of the above-mentioned, the view that Lyme borreliosis is not a separate kind of disease, still persists.

We verified the first cases of Lyme borreliosis in Hungary in 1994, with the help of H. Russell (CDC, USA). In the laboratory of serology at NIH, Hungary we have been carrying out investigations since 1988, with the reagents of the French Diagast and *Borrelia burgdorferi* sensu scripto substrain. These investigations were done with passive haemagglutination after absorption. By April, we determined the reactivity of sera from 100,000 patients.

The statistical reliability of our method: first order error 1.9%, second order error 6.9%.

Positive reports were sent out only after 3 positive results.

Verifications among different laboratories, Prof. Stanek (Vienna), WHO interlaboratory control (1990), EU interlaboratory control (EU Concerted Action on Lyme borreliosis, 1996) are a proof of the reliability of our method.

We have run comparative tests with ELISA kits using whole bacterial antigens, purified antigens, and the recombinant P39-antigen alone and combined with purified antigens.

We can conclude that

Lyme borreliosis is a disease that can be diagnosed serologically, but preliminary absorption has to remove aspecific cross-reactivity, we cannot compare data unless we have an optimal antigen-antibody relation, more seropositivity is obtained when using antigens of more substrains.

The control of the investigations, which may influence the therapy and the chance for curing the patients, has to be regulated by law according to GMP. The modifying

effects of Hungarian substrains have to be determined before introducing any kind of reagent. The facilities for this determination should be provided centrally.

M. FÜZI

Is *Chlamydia pneumoniae* the pathogen of arteriosclerosis?

In 1988 Finnish scientists suggested a link between infection with *C. pneumoniae* and coronariascclerosis with or without myocardial infarction: they could demonstrate antibodies against *C. pneumoniae* significantly more often in patients with these conditions than in healthy controls. These findings were subsequently confirmed by both American and other West European laboratories. Remarkably, besides detecting antibodies against *C. pneumoniae* some groups could also demonstrate the presence of chlamydial antigens and chlamydia derived nucleic acids in sclerotic plaques on blood vessels. It can be considered a breakthrough when in 1995 American scientists cultured living *C. pneumoniae* from the coronaries of a heart transplant patient. Experiments on animals seem also to support the proposal that infection with *C. pneumoniae* and arteriosclerosis are etiologically connected: almost half of the rabbits infected with *C. pneumoniae* through the upper respiratory tract developed sclerotic plaques 7 weeks after infection, while control animals remained normal. Thus, experiments seem to suggest that arteriosclerosis and its aftermath ischaemic heart disease are infectious diseases caused by *C. pneumoniae*.

Author reviews the current stage of research and points out the probable change expected in the therapy of arteriosclerosis.

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Toxin genes, fimbrial attributes and clonality of porcine *Escherichia coli* representing cases of postweaning diarrhoea and oedema disease

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Diarrhoea and oedema disease are two frequent clinical conditions of pigs typically occurring a few days after they are weaned. Both diseases result by enteric colonization due to specific types of *E. coli*, i.e. in case of diarrhoea enterotoxigenic *E. coli* (ETEC), in case of oedema verotoxigenic *E. coli* (VTEC). ETEC produce heat labile (LT) and different heat stable (STa, STb) enterotoxins, while VTEC produce a Shiga-like toxin variant SLT-IIv (VT2e). Colonization of the porcine small intestine by ETEC and VTEC are usually mediated by fimbria (F4 and F18 in case of ETEC, and by F18 in case of VTEC), isolated

from weaned pigs. F18 fimbria occur in two antigenically closely related variants (F18ab and F18ac) (Rippinger et al., 1995).

The aim of these studies was to reveal association of toxin types with fimbrial types and with possible clonal groups among porcine postweaning *E. coli* strains isolated in Austria, Hungary and in the USA.

Bacterial strains comprised 284 *E. coli* strains tested for toxin genotype by DNA hybridization (for LT, ST) and/or by PCR (for SLTII). Fimbriae usually occurring in porcine postweaning strains (F4, F41, F18ab and F18ac) were detected by microfluorescence method using polyclonal or monoclonal antifimbrial antibodies. O serotyping of strains was done by standard methods. A subset of 44 strains was analysed for possible clonal relationship by multilocus enzyme electrophoresis to detect allelic variations at 20 enzyme loci as described by Selander et al. (1986). Genetic relationship among isolates was assessed by groupings inferred from a cluster analysis.

Results of multilocus enzyme electrophoresis applied to 44 Hungarian and US isolates indicated that in general the serogroups and toxin and fimbria types were not related to selected allelic variations (ETs), suggesting that the toxin and fimbrial genes in porcine postweaning *E. coli* isolates occur in a variety of chromosomal backgrounds.

However, in case of serogroups typical for porcine VTEC (O138 and O139) either the genomic diversity was small (O138) or the virulence genes (*sltIIv* and *f18*) were uniform (O139). More diversity in both ET or toxin genotype was observed among ETEC (O141, O147, O157).

VTEC strains producing SLTIIv tended to produce F18ab fimbria while ETEC strains usually produced F18ac. This tendency was also demonstrated on a larger number (240) of postweaning isolates of VTEC and ETEC. In the serogroup most typical for porcine VTEC (O139) the characteristic fimbria were F18ab, while on the bacteria of other – so-called enterotoxigenic – serotypes the fimbria F18ac were far more prevalent. Some enterotoxigenic strains usually produced more than one kind of toxin (STa, STb) and occasionally more than one type of fimbria (F18ac, F4 and/or F41).

GY. VÁMOS

Method of salmonella isolation using BM plates combined with enrichment

The poster is dealing with the further life of the Hungarian Patent. We exhibit the powder form of the plate produced by the firm Merck. The representative results of this method are being under process at this time. As we can see, the method is very simple, practical but an extraordinarily quick one. The selectivity of the plate is much better than the other ones being known till now.

However, according to our opinion, the original ready-made plate have been proved more satisfactory in the practice.

¹GY. GUNICS, ²S. FARKAS, ³J. MOLNÁR

Elimination of antibiotic resistance of *Acinetobacter calcoaceticus* strains by promethazine, imipramine and SDS

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Different concentrations of imipramine, promethazine eliminated various plasmids of antibiotic resistance from 11 *Acinetobacter calcoaceticus* strains.

Elimination of ampicilline, chloramphenicol, kanamycine and tetracycline resistance varied between 1.0–6.1%.

The plasmid curing effect of promethazine was compared to that of acridine orange and SDS on *A. calcoaceticus* strains carrying ampicilline, chloramphenicol, kanamycine and tetracycline resistance. One *A. calcoaceticus* strain had shown segregation in the morphology of colonies and the efficiency of plasmid elimination in the segregants were different.

Z. TIGYI, B. PÁLMAFI, T. PÁL

The effect of congo red on the sensitivity to different colicins

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It has been known that the bacterial cell binding of the Congo Red dye shows correlation with the expression of virulence-related proteins and to the binding of certain molecules, e.g. hemin. We studied the relationship between the presence of Congo Red in the medium and the sensitivity to colicins also interacting with the surface of the bacterial cell. Colicins acting by various mechanisms (Js, E3, D, A and K) were studied in sensitivity assays with and without Congo Red. Our data show that Congo Red interferes with the sensitivity to certain colicins, particularly with that to *Shigella sonnei* colicin Js.

¹K. HÁBER, ¹K. BALOG, ²A. SZENDRŐI, ²M. TAKÁCS, ¹M. MEZEI, ¹Á. LACZA,
¹T. RIGÓ, ²GY. BERENCSI, ¹J. MINÁROVITS

**Cloning of an EcoRI-EcoRI fragment of mycobacteriophage
D29F-DNA into pUC18 vector and analysis of the insert**

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Our earlier phage induction experiments showed natural (poly)lysogenic properties of the different strains of *Mycobacterium smegmatis* (M.sm.b., M.sm.R., SN2) and *Mycobacterium phlei* (Lausanne, SN105, SN109, SN113). Lysogenic state of these strains can be further proved by detecting presence of phage-genoms in the bacterial DNAs. For this purpose we tried to clone restriction fragments of different mycobacteriophage-DNAs into the pUC18 plasmid. The host of the selected phages (D29A, D29F, By and BK1) are different strains of *M. smegmatis*, but phages D29 and BK1 can be propagated on the strains of *M. tuberculosis*, as well.

We present data showing the insertion of an EcoRI-EcoRI fragment (5.8 kb) of D29F phage into the vector. The fragment has two BamHI recognition sequences giving the opportunity for subcloning. Sequencing of the fragment is in progress; at present a 384 bp and a 221 bp are known from the two ends of the insert.

By use of the GenBank database we revealed similarities between the insert and sequences of mycobacterial DNAs and LS mycobacteriophage DNA. The known sequence of the fragment shows 53.0% homology in 304 bp overlap with the gene of major DNA binding protein of rat cytomegalovirus.

D. SZAKÁL, T. PÁL

**Colony blot immunoassay to detect *Shigella*
and enteroinvasive *Escherichia coli* strains in water samples**

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Shigella and enteroinvasive *Escherichia coli* (EIEC) strains are important causative agents of water-born diseases. Detection of the causative agent in water is very difficult with the present routinely used methods. This rather stands for EIEC strains, since their separation from non-pathogenic *E. coli* strains is in most cases impossible using classical microbiological methods.

Our previous results showed that an ELISA based detection of the virulence plasmid coded *IpaC* antigen of *Shigella* and EIEC strains was an appropriate method to identify these pathogens. The aim of the present research was to examine whether this immunological approach using an immunoblot assay was suitable for the detection of enteroinvasive bacteria in water.

Water samples were contaminated with definite amounts of bacteria, then filtered through a nitro-cellulose filter. Filters were placed on media and the resulting colonies were tested by an *IpaC* specific monoclonal antibody. The sensitivity of this colony blot method was compared with that of the *IpaH* specific PCR technique. According to our results this *IpaC* antigen specific membrane blot method is suitable to detect even less than 5 *Shigella* cells in 100 ml water, with 5.000–10.000 non-pathogenic cells in the background.

MYCOLOGY MINISYMPOSIUM: FISSION YEASTS

¹Á. SVEICZER, ¹B. NOVÁK, ²M. MITCHISON

Size control in the cell cycle of the fission yeast, *Schizosaccharomyces pombe*

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The major events of the cell division cycle are coordinated with overall cell growth by size control mechanisms. The cell cycle of wild type fission yeast cells is regulated by the mitotic size control. By an analysis of cell length and cycle time in time-lapse films of the fission yeast we have identified a marker of size control mechanisms: the rate change point (RCP) where the linear rate of length growth increases. The period preceding the RCP is dependent on size (a "sizer") while the period after the RCP is independent of size (a "timer"). We have established that in WT cells the mitotic size control acts much earlier (halfway through G2) than previously believed. We suggest that mitotic size control determines the start of activation of *cdc2/cdc 13* kinase by a tyrosine-dephosphorylation mechanism.

wee 1 and *cdc25* are the major *cdc2* tyrosine-kinase and tyrosine-phosphatase pair, which play antagonistic role in the mitotic control (*wee1* inhibitory, *cdc25* activatory). In *wee1⁻* mutants the mitotic size control is replaced by a G1/S size control which is as strong as the mitotic one. Its RCP is at about the G1S boundary, where the pre-RCP period is a sizer and the post-RCP is a timer again. We show that *cdc25* is not an essential element of the mitotic size control. In the absence of both *wee 1* and *cdc25* (*wee1-50 cdc25Δ* double mutant) the mitotic control is compromised: there is apparently no size control and the cycle times are quantized.

We also show that the G1/S size control is dependent on *rum1*, a stoichiometric inhibitor of *cdc2/cyclin-B* kinases. *Rum1Δ wee1⁻* cells have no size control at all, since the determinants of both size controls are defective: these cells show a shorter cycle time than the mass doubling time, causing a continuous decrease of division length from cycle to cycle.

¹A. CSIKÁSZ-NAGY, ²B. GYÓRFFY, ¹B. NOVÁK, ²J. J. TYSON

Modelling the control of DNA replication in fission yeast

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A central event in the eukaryotic cell cycle is the decision to commence DNA replication (S phase). Strict controls normally operate to prevent repeated rounds of DNA replication without intervening mitoses ("endoreplication"), or initiation of mitosis before DNA is fully replicated ("mitotic catastrophe"). The molecules and interactions that enforce these controls are beginning to be unraveled by elegant physiological, genetic and biochemical studies of fission yeast and budding yeast. We have distilled the evidence derived from *Schizosaccharomyces pombe* into a molecular mechanism of "Start" control. Using established principles of biochemical kinetics, we compare the properties of this model in detail with the observed behavior of various mutant strains of fission yeast: *wee1⁻* (size control at Start), *cdc13 Δ* and *rum1^{OP}* (endoreplication), and *wee1⁻ rum1 Δ* (rapid division cycles of diminishing cell size). We discuss essential features of the mechanism that are responsible for characteristic properties of Start control in fission yeast, in order to expose our proposal to crucial experimental tests.

¹I. MIKLÓS, ¹Á. GRALLERT, ¹E. ZILÁHI, ^{1,2}M. SIPICZKI

Genetic and cytological analysis of a cytokinesis mutant in *Schizosaccharomyces pombe*

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Schizosaccharomyces pombe, the eukaryotic unicellular fission yeast is an attractive model organism of the study of cell cycle control. A lot of genes are known in this process, however many of them are involved in the S-phase or in mitosis. The very last step of the cell division, cytokinesis, is poorly understood from genetic point of view.

That is why we decided to isolate and analyse new mutant strains, which show abnormality in cytokinesis. The *spl 1* strain belongs to the one group of that mutants. It shows the following phenotype at restrictive temperature (35 °C):

The *spl 1* mutant strain produces threads consisting of 2 or 4 cells, which do not separate from each other. The cells are frequently bent. The cells are not able to split their septa. As the electron microscopic photos suggest it can not be attributed to the structural abnormalities of the septa. The fluorescent staining of the cells showed a defect in the position and shape of the nuclei, in the localization of actin dots and in the appearance of spindle apparatus. The cloning of the gene is under way. It can be found on a 4kb DNA fragment

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Fission-yeast cytokinesis mutants defective in cell differentiation

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In a screen of a UV-mutagenized *Schizosaccharomyces pombe* homothallic population we isolated sterile mutants showing incomplete or partially defective cytokinesis. Due to the impaired cytokinesis (frequent lack of cell separation after the completion of septation), the mutants formed chains of non-separated cells resembling the true mycelia of *sep1-1*. The genetic analysis (complementation and recombination) classified the mutants into 11 groups which define 11 novel *sep* genes (*sep6-sep16*). The electron-microscopic and calcofluor-stained fluorescence images of the mutants did not show abnormal septal structure, and the immunofluorescence staining of the actin and tubulin cytoskeleton did not show any remarkable changes when compared to the wild type. All mutants were defective in conjugation, and most of them were also defective in meiosis and sporulation except the groups designated *sep6* and *sep12*. The inactivation of *pat1*, a negative regulator of sexual differentiation (the mutant allele *pat1-114^{ts}* induces conjugation and sporulation at the restrictive temperatures regardless of the physiological conditions) provoked sporulation in 7 groups indicating that those mutants are defective upstream of *pat1* in the sexual pathway. *Sep6*, *sep8*, *sep11* and *sep16* remained sterile even at the *pat 1-114* background suggesting that the affected genes act downstream of *pat1⁺*. The fluorescence staining of the nuclei revealed a defect of chromosome separation in *sep9*, *sep13* and *sep15* mutants. When the tubulin cytoskeleton and the SPBs (equivalents of the centrioles of higher eukaryotes) were stained simultaneously by immunofluorescence techniques, we observed cells showing interphase cytoskeleton with duplicated SPBs in certain mutants. These mutants are most probably defective in the correct timing of disassembly of the cytoplasmic cytoskeleton and the initiation of spindle formation.

^{1,2}E. ZILÁHI, ²Á. GRALLERT, ^{1,2}M. SIPICZKI

Investigations of the *sep15* gene of *Schizosaccharomyces pombe*

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Schizosaccharomyces pombe is an eukaryotic haploid organism. Fission yeast cells grow by elongation at their ends and divide by binary fission after forming a centrally placed septum.

We have identified a number of cytokinesis mutants called *sep/sp1* mutants. *sep* mutants are able to form septa, yet they are unable to complete cytokinesis. Since the daughter cells do not separate, the mutants form short mycelia. The phenotype of the

mutants might result from various defects. These mutants fall into four classes; *sep1* encodes a transcriptional factor, *sp11* is defective in spindle formation as well the position and the shape of the nucleus are abnormal, *sep2-5* mutant cells show resistance against cell wall lytic enzymes and they are defective in septum formation, *sep6-16* mutants are aberrant in cell differentiation which causes the sterility of the cells.

We were particularly interested in *sep15* mutant which is sterile in addition to displaying the *sep* phenotype.

It was established using classical genetic methods that the inheritance of *sep* phenotype and sterility is a recessive feature encoded by the same gene. We tried to find out any functional interaction creating double mutants between the *sep15* and other *cdc* (cell division cycle) mutants.

It was found using immunofluorescence techniques that the distribution of the actin cytoskeleton and tubulin is normal but the separation of the chromosomes is aberrant at restrictive temperature.

In addition to the classical genetic methods we have cloned the *sep15* gene. The phenotype of the *sep15* cells made us to develop a direct selection system (the cells are not able to form colonies at restrictive temperature) for the cloning. We used conventional genomic genebanks called pSR1/pSR2 for the selection. As a result of the cloning we have found a 1.5 kb fragment which carried the *sep15⁺* information. After the physical mapping of this fragment we started the subcloning of it using pUR18N/pUR19N shuttle vectors to define the gene providing the above-mentioned functions.

After determining the DNA sequence of *sep15* gene, additional molecular investigations will be needed to reveal what process the gene is involved in. The complete characterisation and the findings of cytological and genetic analysis of the *sep15* gene may give further understandings of the regulation of cytokinesis.

¹Zs. BENKO, ^{1,2}M. SIPICZKI

Study of effect of caffeine on cells using pleiotropic multidrug resistant mutants

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Caffeine is one of the most frequently consumed drug. Several aspects of effect of caffeine on living organisms are studied. The latest results showed that caffeine could not cause cancer alone but it could increase effectively the carcinogen effect of several compounds. In addition caffeine is a well-known toxin of cells with different effects on cells. The most common changes in phenotype following addition of caffeine is the prolonged cell cycle and formation of elongated cells.

To understand these effects we isolated caffeine-resistant mutants that could tolerate 4–5 times higher concentration of caffeine than the wild-type cells. One hundred mutants isolated were brought into four complementation groups. One representative was chosen from each group for further study. Analysis of these mutants revealed several

dissimilarity to wild-type cells such as prolonged cell cycle, elongated cells, UV sensitivity, γ -resistance, pH-sensitive growth, damaged meiosis, starvation sensitivity and other drug resistance. These results suggest that a very complex system exist in the cells that is able to reduce the toxic effect of caffeine. To our mind only one common biochemical pathway should exist which a mutation can reduce the toxic effect of caffeine. We also found that *S. pombe* cells could adapt to higher concentration of caffeine. This adaptation is reversible. It seems to be reasonable that there is a relation between the mechanism of adaptation and the genes that could cause caffeine-resistance by mutation. To understand this phenomenon more clearly we have cloned three of the four *caf* genes and sequenced them. Molecular study shows that the gene product of *caf2* (*crm1*) is responsible for formation of compact chromosome structure. Deletion of this gene is lethal for cells. The *caf1* (*hba1*) gene product is located in the nucleus and seems to be a new type of protein. The *caf4* (*trr1*) gene encodes thioredoxin reductase enzyme. The cloning of the fourth *caf* gene is in progress now. Cells containing the disrupt alleles of *caf1* or *caf4* genes get caffeine sensitive as wild-type cells and the generation times prolong extremely depending on the pH of medium. The *caf* genes interact with several different genes. For example the mutation of the *caf2*(*crm1*) gene causes caffeine-resistance through the *pap1* gene and the p25 protein. In addition there is interference between the *caf1* and *caf2* genes. A mutation in the *caf4* (*trr1*) gene can cause inhibition of the effect of the wild-type p53 human protein that inhibits the cell growth in *S. pombe*. The *caf3-89* dominant mutation increases the morphological changes in phenotype caused by *sep1* and *ste1* while the *caf3-89sep13* double mutation is lethal. The *caf3* gene even interacts with some auxotroph gene.

Mutations in *crm1*(*caf2*, *bar1*) can even cause resistance to brefeldin A (an antifungal antibiotic that inhibits the Golgi complex), staurosporin (inhibits the protein kinase C) and leptomicin B (an antifungal antibiotic that inhibits cell cycle arrest). The wild-type allele of *hba1*(*caf1*) can cause brefeldin A resistance on high copy plasmid.

¹K. CZAKÓ-VÉRI, ²M. BATIC, ²M. PAS, ¹M. PESTI, ³M. SIPICZKI, ²P. RASPOR

How *Schizosaccharomyces pombe* mutants accumulate chromium

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Cr(VI) sensitive and tolerant mutants of *Schizosaccharomyces pombe* have been isolated after NTG and UV mutagen treatment from a heterotrophic auxotroph strain, designated *lys1-131h⁺*.

Freshly prepared sterile K₂Cr₂O₇ solution was applied as Cr(VI) into YES media, (0.5% yeast extract, 3% glucose plus 100 mg/litre lysine) or into IPES puffer.

The accumulation studies were carried out in BIO/CHEMUP bioreactor with ShivaI/BIA software. The changes of the temperature, pH, pO₂ and the biomass were

registrated. The chromium content of the biomass was determined with atomic absorption spectrometry, typ. VARIAN AA-575. The biomass were treated with HNO_3 for "total chromium" (ToCr) while with NH_4OH for "organic bound chromium" (OBCr) analysis.

I. The chromium accumulation of the examined strains has been measured in YES. At the late log-phase 75 μM Cr(VI) was given to the cultures. Samples were taken after 5 minutes and after each 2 hours.

The chromium accumulation was the highest in the cells of the sensitive mutant strain. After the first 5 minutes the ToCr was 800 $\mu\text{g/g}$ dry biomass(dbm), after 2 hours 880 $\mu\text{g/g}$ dbm and after 16 hours 930 $\mu\text{g/g}$ dbm, respectively. During the same time the parental strain accumulated 190, 470 and 850 $\mu\text{g/g}$ dbm, the tolerant mutant strain 120, 340 and 620 $\mu\text{g/g}$ dbm chromium. The ratio of the ToCr and OBCr was 2:1 at mutants and 3:1 at the parental strain.

II: For the investigation of the cell wall absorption, the chromium accumulation of the three strains were determined in PIPES puffer (pH=6.0). The washed biomass was suspended in puffer, containing 75 μM chromium (VI) for two hours. One half of the samples were washed only with PIPES, the other half with PIPES than with EDTA (pH=4.0).

Results showed in both experiments that the chromium accumulation of the mutant strains was significantly higher than that the parental one. After washing procedure with EDTA significantly decreased the ToCr and OBCr of the parental and tolerant strains, in contrast to the sensitive one, where the difference was only 40 $\mu\text{g/g}$ dbm proving the significantly different chromium metabolisms of the mutants.

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The role of cell surface molecules in the flocculation of *Schizosaccharomyces pombe* rive 4-2-1 strain

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Yeast flocculation is a common cell-aggregation phenomenon occurring among different yeast species. Cells of *Schizosaccharomyces pombe* RIVE 4-2-1 strain flocculated in shaken culture in media containing 5% of ethanol at the late exponential phase of growth.

Flocculation was studied in bioreactor using various culture media and wine, at different concentrations of ethanol under aerobic and anaerobic conditions. Bioreactor controlled by a computer program and connected to a pump and photometer was used for on-line measurement of flocculation. Ethanol addition into the culture media was necessary at the lag phase of the growth to induce flocculation. We obtained high flocculation rate when the inoculum was also flocculent. Under anaerobic circumstances

the growth and flocculation were not satisfactory. In the culture media glucose could be replaced by saccharose, it was not disadvantageous for growth and flocculation.

Surface structure of flocculent and non-flocculent cells was studied by scanning (SEM) and transmission electron microscopy (TEM), which revealed an amorphous, slime-like or fibrillar structure of the outermost cell surface. Close contact of the adjacent cells was found, however, only in the case of flocculated cells.

Treatment of cell clumps with proteolytic and cell wall lytic enzymes caused quick disaggregation of cells. TEM pictures showed partial or complete digestion of the cell wall.

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^{1,2}M. SIPICZKI, ¹I. MIKLÓS, ¹A. GRALLERT

Dimorphic fission yeasts

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The fission yeasts are unicellular ascomycetes which divide by septation and fission near the middle of their more or less cylindrical cells. *Schizosaccharomyces pombe* and *S. octosporus* form only yeasts cells, whereas *S. japonicus* is a dimorphic organism which has both a yeast and a mycelial phase. In liquid media only yeasts cells are formed, but on solid media the mycelial morphology is also common. The yeast-mycelial transition is associated with a switch from the bipolar growth to a unipolar extension and an intense vacuolation at the non-growing cell end. The process is triggered by the decrease of intracellular cAMP level. The mycelium is prone to undergo arthrosporogenesis and can also return to yeast phase if the level of cAMP is increased by caffeine or by a shift to 35 °C. The distribution actin is less regular in the yeast cells than in *S. pombe*, but accumulation of actin dots are visible at the growing and the site of septation (actin ring). In the mycelial cells actin is visible in the whole cytoplasm, although most of it is concentrated at the growing hyphal tips. The dimorphic cycle of *S. japonicus* offers a convenient model for the investigation of polar cell growth and the regulation of dimorphic transition characteristic of many fungal species

MYCOLOGY AND INDUSTRIAL MICROBIOLOGY

¹E. SÁNDOR, ¹L. KARAFFA, ¹I. PÓCSI, ¹E. FEKETE, ²J. KOZMA, ¹A. SZENTIRMAI

**Is there any casual relationship between fragmentation
and cephalosporin-c production of *Acremonium*
chrysogenum?**

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The production of secondary metabolites is a typical two-stages process since the trophophase and the idiophase are separated in time. In the case of the filamentous fungi *Acremonium chrysogenum*, which is a well-known producer of cephalosporin-C (CPC), these stages are characterised with different morphological forms. Here we present experimental data whether there is a casual relationship between the observed morphological changes and the CPC biosynthesis.

In the trophophase of batch cultures, mycelia were characterised with filamentous morphology while in the idiophase fungal cells showed a tendency to differentiate into wide, swollen, yeast-like forms. Comparing the time-course of the morphological changes observed with high (4–5 g/l) and low (0.4–0.5 g/l) producer strains, we established that the higher CPC production always coincided with an enhanced fragmentation during the idiophase.

Different cell-wall degrading hydrolases, e.g. chitinase(s), might play a crucial role in the observed morphological changes. In fact, the specific activity of chitinase(s) increased throughout the fragmentation. Regarding that in the presence of allosamidin, a potent and specific inhibitor of chitinase, the fragmentation was inhibited significantly, but no concomitant change in the CPC production was observed, we assumed that there was no casual relationship between the fragmentation and the CPC production.

In further experiments we studied chemostat cultures, where the ratio of yeast-like cells was highly dependent on the dilution rates, which, at the same time, had no effect on the CPC production. Hence, we concluded that there was a casual relationship between the growth rates and the fragmentation of the cultures, but a similar direct connection between fragmentation and antibiotic production was finally excluded.

¹É. SUGÁR, ¹M. DÉN, ¹K. URBÁNSZKI, ¹G. SZAKÁCS, ²R. P. TENDERDY
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Cellulase and xylanase production on corn fiber by solid substrate fermentation

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About four million tons/year of corn fiber is produced in the USA alone in wet milling processing. This vast resource may be converted into valuable products such as ethanol and animal feed by simultaneous enzymatic saccharification and co-fermentation by yeast (SSCF). The necessary enzymes may be produced economically by in situ solid substrate fermentation (SSF), as part of the overall process. In laboratory scale SSF, 5 g dried corn fiber was supplemented with mineral salts and nitrogen source, inoculated with 10^7 spores of lignocellulolytic fungi/g substrate, and fermented for eight days in 750 ml Erlenmeyer flasks at 75% moisture content and 20 °C. The best results were obtained with *Trichoderma reesei* Rut C30 (7.1 FPU/g Dry Weight (DW) cellulase activity, 4.9 IU/g DW beta glucosidase activity, and 1150 IU/g DW xylanase activity), but other fungi were. The recommended cellulase loading rate in the SSCF process is 0.75 FPU/g corn fiber DW, therefore a 1:10 mixing ratio of in situ enzyme and corn fiber would satisfy the requirements, at an estimated enzyme cost of about \$ 2.4/MT, also suitable compared with estimated enzyme cost of \$ 15/MT in submerged fermentation. The in situ produced enzyme is thus an economic source for the bioprocessing of corn fiber.

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Denitrification (N₂O-release) by *Streptomyces nitrosporeus* in aerobic systems

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The amount of nitrous oxide produced from nitrate by *Streptomyces nitrosporeus* DSM 40023 was determined using in vitro model experiments by incubating for 14 days under standard conditions (28 °C, 100 rpm, pH 6.5–7.0, initial atmospheric O₂ concentration). Specific inhibitors (antimycin-A, oligomycin, 8-hydroxyquinoline, sodium chlorite) were used for the identification of crucial points in the pathway. It is assumed that *Streptomyces nitrosporeus* can use nitrate and nitrite as an acceptor in electron transport chain, and so maintains the respiration.

Use of the Acetylene Inhibition Technique (AIT) confirmed that the end-product of the process is not N₂ but N₂O, what is slightly harmful in environmental terms.

Beside the oxygen concentration, both the pH of the medium and the nitrate concentration significantly affected the amount of N₂O produced in the course of the

nitrate reduction. *S. nitrosporeus* did not produce N_2O under anaerobic (no growth) or under aerobic (21% v/v O_2) conditions. However, at decreased partial pressure of O_2 (at 5 or 10% v/v), the amount of N_2O formed increased to $5.2 \mu g\ ml^{-1}$ after 14 day incubation period. The amount of N_2O produced in slightly acidic medium (pH 6) was 20 times higher than that at neutral pH and 200 times higher than in an alkaline nutrient solution (pH 8). A 50% decrease in nitrate concentration reduced the production of N_2O by 90%.

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The most often occurring moulds identified at the storage of grain of *Helianthus annuus*

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The grains of sunflower (*Helianthus annuus*) are rich of oil. The quality of vegetable oil depends on storage of harvested grains. Sometime the moulds can cause spoilage, when the water content of the grains is between 10–20%. For this reason a model system was controlled because of mouldy for 6 weeks. The sunflower was prepared with 10 and 20% of water content, at 25 °C. Before storage the most often occurring species belonged to *Alternaria alternata*.

First of all the *Aspergilli* were able to grow during the time of incubation. Morphology of the colonies and typical characteristics of scanning electron micrographs were used for identification of moulds. After 6 weeks strains of *Aspergillus parasiticus* could be isolated and demonstrated as a dominant species. However the production of aflatoxin was not detected in case of *A. parasiticus* at the model storage at the environmental conditions used.

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Altered mitochondrial genome organisation in the species *Aspergillus carbonarius*

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Aspergillus carbonarius is the most distinct member of the black *Aspergilli* (section *Nigri*) exhibiting some unique phenotypical and molecular characters. On the bases of RFLP studies two molecular groups (types 1 and 2) could be distinguished among examined strains [1]. The mtDNA type 1 could be divided into two subgroups (types 1a and 1b) [1]. The sizes of mt genomes of *A. carbonarius* strains (types 1a: 61.2; 1b: 62.2; type 2: 42.7 kb) are 10–30 kb larger than those of belonging to *Aspergillus niger* aggregate [2].

For interpretation of these size differences, restriction maps were constructed by several enzymes. Isolated mtDNA was digested with *EcoRI* and the fragments were separated by agarose gel electrophoresis. These fragments were either redigested with various enzymes, or were cloned into pBluescript KS plasmid. The clones were digested with the following enzymes: *BamHI*, *EcoRV*, *HhaI*, *KpnI*, *PstI* and *XhoI*. On the basis of these data linearized physical maps were developed. Digoxigenin-labelled hybridisation probes (derived from the *Aspergillus nidulans* mt genome [3]) were applied for identifying the possible positions of known coding regions.

Restriction and functional maps were constructed with six enzymes in each case. It was proved that certain NADH dehydrogenase subunits of *A. nidulans* mt genome (*ndh2*: A3, A4, A5) are absent from mtDNAs of all *A. carbonarius* strains. The *ndh2*: A6 gene of *A. nidulans* is also absent from mtDNA type 1b, but is present in types 1a and 2. The 1 kb size difference between 1a and 1b mtDNA types could be localised to a *BamHI* fragment. The nearly 20 kb difference between types 1 and 2 may be due to the presence or absence of intergenic regions.

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É. KEVEI, E. RINYU, B. TÓTH, J. VARGA

Detection of double-stranded RNA Mycoviruses in species of *Aspergillus* section *Circumdati*

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Sixty strains belonging to *Aspergillus* section *Circumdati* were tested for the presence of double-stranded RNA (dsRNA) mycoviruses. Five of the 27 *Aspergillus ochraceus*, and 1 of the 5 *Petromyces alliaceus* strains were found to contain dsRNA fragments. The 28 other strains belonging to this section were not infected, so altogether 10% of the examined *Aspergillus* section *Circumdati* strains harboured dsRNA elements. This ratio is similar to the frequency of virus-infected cultures found in *Aspergillus* section *Nigri* (Varga et al., 1994), and section *Flavi* (Elias and Cotty, 1996). The double-stranded nature of these RNA fragments was confirmed by RNase and S1 nuclease treatments. The stromata of some mycovirus containing strains were examined for their ability to transmit the dsRNA elements. Asci of the sexual *P. alliaceus* species are produced within such stromata. Single stromata from the virus-infected *P. alliaceus* strain were isolated and cultivated. All single-stromatal cultures lacked a 3.5 kb dsRNA band which was present in the original isolate, while a 0.6 kb dsRNA fragment was present in all of these cultures. The examination of the isolated mitochondrial fraction suggests that this 0.6 kb dsRNA fragment is probably located in the mitochondria of these strains.

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Genome organization of a new mycovirus from *Fusarium poae*

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Geographically different, vegetatively incompatible strains of *Fusarium poae* were found to harbour virus-like particles with double-stranded RNA genome; the number of these elements ranged from 1 to 12 among strains with sizes between 0.4 and 12.0 kb. RNA/RNA hybridization experiments were used to determine the genetic relatedness of the various dsRNA elements. According to the hybridization patterns, mixed virus infections could be demonstrated in most strains of the fungus and the similar-sized fragments present in vegetatively incompatible strains proved to be homologues. Virus-like particles, 25 nm in size were purified from A-11, a strain containing five dsRNA bands; when nucleic acids from these particles were subjected to electrophoretic separation, only two of the bands, sizing 2150 and 550 bp, respectively were recovered indicating that the other three fragments are maintained in unencapsidated forms in the fungal cells. The encapsidated dsRNA fragments were converted to cDNA clones by reverse transcription and the clones were subjected to sequence analysis. These experiments showed that the larger, 2150 bp band of the virus comprises two co-migrating dsRNA fragments: one of them encodes an RNA dependent RNA polymerase, while the other contains the genetic determinants of the coat protein. The genome organization and certain sequence motifs of this new mycovirus, named FPVI show striking similarities to that of the *Atkinsonella hypoxylon* 2H virus, a member of the *Partitiviridae* family.

J. KUCSERA, A. GÁCSER and I. PFEIFFER

Comparative analysis of killer properties of *Saccharomyces daiirensis* and *Saccharomyces cerevisiae*

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Among *Saccharomyces cerevisiae* killer strains three classes are discriminated: K1, K2 and K28. Members of the groups produce proteinaceous toxins which kill non-killer yeasts, and killer yeasts belonging to opposite killer classes. All three killer toxins are

encoded by different dsRNA molecules (M1, M2, M28) which are encapsidated into virus-like particles (VLP). All killer strains contain another dsRNA molecule (L-A) encapsidated also into VLP-s. L-A codes for the capsid protein and dsRNA dependent RNA polymerase.

Previously we have found that *Saccharomyces dairensis* CBS 421 has killer character [1] and is encoded by nuclear gene(s). Biochemical properties of its toxin protein were compared to the known types of *S. cerevisiae*. The results showed differences in their killing spectra, but similarities in pH optima and the mode of toxin action, established by bromocresol purple staining. On the basis of these similarities we supposed that the killer phenotype might be caused by a *S. cerevisiae* like virus which integrated into the chromosome, following a reverse transcription. To test this possibility cDNA copies of the K1 and K2 toxin genes were hybridised to *S. dairensis*. They showed no homologies to the total DNA of *S. dairensis* CBS 421.

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¹Zs. ANTAL, ²L. MANCZINGER, ³Gy. SZAKÁCS, ⁴R. P. TENGEDY, ^{1,3}L. FERENCZY

Investigation of the antagonistic potential of cold-tolerant *Trichoderma* strains against plant-pathogenic fungi

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The cold tolerance of 360 *Trichoderma* strains isolated from fourteen Hungarian soil and leaf litter samples or from North-American soil were examined. The strains represented six different species groups of the *Trichoderma* genus. Fourteen strains were selected which exhibited considerable growth rates at 5 °C both on minimal and yeast extract media.

The antagonistic properties of these strains were examined against the plant parasitic *Pythium debaryanum*, *Fusarium oxysporum* f.sp. *dianthi* and *Rhizoctonia solani* as test organisms both on minimal and yeast extract media. The activities of the extracellular chitinases and proteinases, which are thought to be involved in the mycoparasitic process were also examined. These enzymes were highly active at 10 °C in the six strains which proved to be the most potent antagonists of the above-mentioned economically important plant parasites.

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Transformation of *Trichoderma hamatum* with the *Tham ch* gene results in various types of integration and increased levels of chitinase activity

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An endochitinase encoding gene, *Tham-ch* was cloned by screening the genomic library of *Trichoderma hamatum*, strain Tam-61 with a PCR-amplified chitinase sequence from the same fungus. PEG-mediated homologous transformation with *Tham-ch* under selection pressure provided by hygromycin-B resulted in a transformation frequency exceeding 300 colonies/ μ g DNA. Eleven stable transformants were tested for chitinase activity using the fluorogenic substrate, 4-methylumbelliferyl β -D-N,N'-triacylchitotriose. Chitinase activity significantly increased in all but one transformants. Integration of the transforming construction was demonstrated by Southern blotting. Homologous single copy integration occurred in eight transformants, homologous tandem integration was observed in two transformants, while non-homologous tandem integration was detected in one transformant. No correlation was found between integration patterns and changes in the chitinase activity of the transformants. The aggressiveness of potential biocontrol strains could successfully be improved by increasing the copy number of chitinase encoding genes.

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Cr(VI) induced alterations in yeast plasma membrane

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The effect of $K_2Cr_2O_7$ on the ergosterol-producing wild-type strain, designated as 33 *erg*⁺ (ATCC 44829) and its mutant deficient in ergosterol, designated as *erg* 2 (ATCC 44831) of the human pathogenic eukaryotic yeast, *Candida albicans* were studied using electron paramagnetic resonance (EPR) spectroscopy to monitor the fluidity of the plasma membrane by the spin labelling method. EPR spectra of spin labels 5-SASL and 14-SASL (fatty acids labelled at the 5th and the 14th carbon atom from the head group with the dimethyl oxazolidine nitroxide) were recorded in temperature range between 0–30 °C on both control and chromium treated (0.6 mM Cr(VI) for 10 min or 120 min) protoplasts. Minimal inhibitory concentration of Cr(VI) in shaken liquid minimal medium proved to be 0.6 mM at 30 °C for both strains. For EPR study, protoplasts were prepared by snail enzyme treatment and osmotically stabilized with 0.6 M KCl.

Both spin labels incorporated into the membrane of the protoplasts reflected different mobilities depending on strain: Ergosterol-producing wild-type strain 33 *erg*⁺

exhibited larger fluidity in comparison to mutant *erg-2*; the hyperfine splitting $2A'_{zz}$ characterizing the mobility of spin labels was smaller for samples of strain *33 erg⁺* in the whole temperature range studied. At 16 °C and 19 °C (*erg-2*) small phase transitions were detected in control samples of both strains. Treatment with Cr(VI) induced decrease of the phase transition temperatures by about 6 °C and new phase transitions appeared for both strains at 20 °C.

Spin labels 14-SASL where the nitroxide moiety is located in the more hydrophobic environment of the membrane reported significant difference between strain *33 erg⁺* and *erg-2*; addition of Cr(VI) produced increase of rotational mobility. The order parameter (S) calculated from the spectra showed phase transitions at 12 °C and 15 °C, respectively.

After preparation an EPR signal arising from Cr(V) was detected evidencing rapid reduction of Cr(VI) to Cr(V). The double integral of the newly formed Cr(V) species decreased in time which is due to chemical reactions. The spectral change of spin label signal was also observed which is very likely the consequence of metabolic activity in the membranes.

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Investigation of the adhesion of *Candida albicans*

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The yeast *Candida albicans* can often be detected in the mouth cavity adhering to the buccal epithelial cells and to the surface of teeth or denture. This can be the beginning of a fungal infection.

We have made in vitro experiments in order to see how chlorhexidine (chlorhexidine is active ingredient of Corsodyl) can be used to remove yeast cells adhered to buccal epithelial cells or to surfaces of acrylates.

We have found, that 0.1–1 µg/ml solution of chlorhexidine very efficiently (30–100%) inhibited the growth of *C. albicans* in batch cultures.

This concentration of chlorhexidine used only once can remove 50–80% of the adsorbed yeast cells from surfaces of acrylates of different origin. Similar results can be achieved by the use of H₂O₂ or UV light. *C. albicans* cells can be completely removed from the surfaces of acrylates if we use 1–2 mg chlorhexidine/ml.

We have investigated in vitro the adhesion of two different fluconazole sensitive (MIC_{fluconazole} = 5 µg/ml and MIC_{fluconazole} > 80 µg/ml) *C. albicans* strains to buccal epithelial cells [1]. We have found that fluconazole resistant yeast cells adhere more than three times better to these cells than fluconazole sensitive ones. The adhesion of yeast cells to buccal epithelial cells does not change significantly at low (0.2 µg/ml)

chlorhexidine concentration. We have found that In the presance of fluconazole (3 µg/ml and 30 µg/ml) the number of adhered *C. albicans* ($MIC_{\text{fluconazole}} = 5 \text{ µg/ml}$) cells were always greater than in case of control.

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ZS. HORVÁTH and J. ZALA

Evaluation of a comparison of the air sampling methods in mycology

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The aeromycological examinations for the purpose of hospital hygiene and allergology require that the adopted methods make clear the limits in principle as to the advantages and disadvantages of each method.

Among the cultivation methods the Koch-type sedimentation method is not suitable for the estimation of the quantitative fungi content in the air. However the sampling methods based on the volumetric principle for the cultivation of fungi from air were invented for the correct appreciation of the real quantitative fungi content, but the method based on the Hirsh-principle morphological examination (Burkard-trap method) gives considerably higher reading for the fungi. The latter method for the quantitative purpose is correct and unique from the point of view that it could show the dead fungi particles as well, which could have an allergological significance. On the other hand all cultivation methods make reduction in the total number of the living fungi particles for the following reason:

1. fungi occur in groups but only one colony forming unit; 2. the slowest growing fungi are suppressed by the quickest growing ones; 3. some of the fungi have a special supply-requirement, therefore they cannot grow in the usually common supply used.

Despite giving a correct result in respect of quantity the Burkard-trap method has some disadvantages, as follows:

1. the spectrum of the nonidentifiable fungi is very wide because of the common occurrence of simple conodio-, and sporogenesis among fungi; 2. the pieces of mycelia are not identifiable according to this method, as mycelia belonging to the identifiable fungi reduce the real number of those groups; 3. fungi which have a tetroconidial propagation, in the process of the developing conidia have the simplest morphological character/shape therefore the group according to this method cause their youg-conidia to reduce the ratio of this group among fungi.

The parallel use of the two methods based on two different principles (the cultivation method using a volumetric air sampler and the Hirsh principle sampling by means of the same instrument) and the critical estimation of the results could give more correct information about the quantitative and qualitative fungi content of air.

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Aeromycological examination of the hospital cave of Tapolca, Hungary

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The aim of our investigations was to study the fungal flora in the cave of Tapolca and in the open air near the cave.

The examinations were carried out with two sampling methods on five fungal media at 10 inner (cave) and 2 outer (surface) sampling points. The 5 sampling times were in January, March, April, September, October.

In the air of the cave there were fungal species – being able to be cultivated – as follows: *Alternaria alternata*, *Aspergillus niger*, *Aspergillus penicillioides*, *Aspergillus terreus*, *Cladosporium herbarum*, *Cladosporium cucumerinum*, *Cladosporium musae*, *Cladosporium chlorocephalum*, *Cladosporium sphaerospermum*, *Cladosporium spongiosum*, *Penicillium inflatum*, *Penicillium hirtusum*, *Penicillium chrysogenum*, *Penicillium variable*, *Penicillium glabrum*, *Penicillium griseofulvum*, *Penicillium funiculosum*, *Rhizopus nigricans*, sterile mycelia I., II., III.

There were a wider range and variety of flora – being able to be cultivated – at the control points outside the cave: *Helminthosporium* sp., *Drechslera* sp., *Paecilomyces variotii*, *Trichoderma viride*, *Acremonium* sp., *Rhodotorula* sp., *Sporobolomyces* sp., *Cryptococcus albidus*, *Mucoraceae*/fam. sp., *Moniliaceae*/fam. sp., *Monilia sitophila*, etc.

Usage of the cave for therapeutical purposes cannot be recommended due to both the high fungal content and the presence of potential allergenic species with generally high CFU (Colony Forming Unit).

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Regulation of the cyanide-resistant alternative respiration in *Acremonium chrysogenum*

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One of the characteristic features of plant and fungal mitochondria is the possession of a cyanide-resistant, hydroxamic acid sensitive, non-phosphorylating alternative respiration. This path is present in *Acremonium chrysogenum* as well. In this lecture, the factors modulating the alternative respiration and also the physiological background of their effects will be discussed.

The specific growth rate is the basic parameter of any microbial population. Unlike most biochemical processes, the alternative respiration inversely correlates with it. This

verifies the observation that the relative activity (engagement) of the alternative path reaches its maximum at the stationary phase of growth.

Alternative respiration is also modified by the quality of carbon source. In this respect, plant oils are much more beneficial than glycolytic ones. According to our hypothesis, the oil degradation, unlike that of glucose, is essentially out of the cell's control. Therefore, the enhanced level of Ao-CoA overloads the Krebs-cycle, which, in this case, needs the exempting operation of the alternative path.

The oxygen supply is of crucial importance in the regulation of the alternative path. The K_m for oxygen is one magnitude higher in the case of alternative oxidase compared to the cytochrome one, therefore it operates at high oxygen tensions only. All the industrial fermentations in which the alternative respiration plays a role (e.g. citric acid, cephalosporin-C, fusidinic acid) need high oxygen tensions, while interruptions in aeration coincide with reduced productivity.

The role of nitrogen source is also significant. On nitrate, both the capacity, which indicates the amount of the component of the alternative path, and also the activity, corresponds to the in vivo electron transport via this path is 15 times higher than those on ammonium. On ammonium-nitrate, the values are on a low level, indicating the possible connection of nitrate reduction and alternative respiration.

The amount of phosphorus present in the medium is also important, which, given the non-phosphorylating feature of the alternative path, is easy to understand.

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Growth, morphology and cephalosporin-c production of *Acremonium chrysogenum* in high cell density cultures

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There are two main reasons for the use of dialysis membranes in microbial reactor systems. First, dialysis membranes are very efficient for continuous removal of inhibitory or toxic products during the fermentation, and second, microbial cultures can be supplied with substrate via the membrane. In this way, high concentrations of microorganisms can be achieved because the flow of substrate is uncoupled from the removal of microorganisms from the fermentor, and the volume of the broth remains constant – unlike in continuous and fed-batch systems, respectively. With the use of the membrane, one can avoid the shortcomings of the conventional, two-vessel dialysis systems like difficulties in sterilisation and mechanical stress on living cells during the pumping procedure.

In this study, the growth, morphology and cephalosporin-C (CPC) antibiotic production of the filamentous fungi *Acremonium chrysogenum* was investigated in high cell density cultures with the use of a Bioengineering dialysis reactor.

In conventional batch systems in minimal medium containing glucose as a sole carbon source the yield coefficient ($Y_{X/S}$) for glucose is about 0.32–0.38, which declines steadily above 6 per cent glucose concentration. Under dialysing circumstances, yield coefficient has not changed, but, due to the nutrient supply from the outer chamber, a significantly higher biomass (28 g/l) could be achieved with a 2 per cent inner and 2 per cent outer initial glucose concentration. Specific growth rate of the culture ($D=0.06\text{ h}^{-1}$) was equivalent with the value measured under batch conditions.

In the exponential phase of growth cells exhibited an extraordinary filamentous morphology, while in the stationary phase cultures were dominated by yeast-like cells.

Ratio of the CPC and biomass production was the same in batch and in dialysis systems, which indicates that the CPC productivity is independent of the cell density.

T. EMRI, I. PÓCSI and A. SZENTIRMAI

**Glutathione probably regulates the intracellular
phenoxyacetic acid concentration in *Penicillium*
chrysogenum under β -lactam producing conditions**

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In a previous study we demonstrated that phenoxyacetic acid (POA; side-chain precursor of penicillin V) depleted the glutathione (GSH) pool and increased the specific glutathione-S-transferase (GST) and γ -glutamyltranspeptidase (γ GT) activities, i.e. the enzyme activities of the GSH-dependent detoxification, in *Penicillium chrysogenum* [1]. Here we present some experimental data on the changes in the intra- and extracellular POA concentrations and on the effect of silibinin (a potent inhibitor of GST), 1-chloro-2,4-dinitrobenzene (CDNB; a substrate and inducer of GST) and the mixture of borate and L-serine (an inhibitor of γ GT) under penicillin producing conditions.

During a 90 h fermentation no change in the extracellular POA concentrations was observed until the penicillin production had started at 60 h, but it decreased continuously later on. The intracellular POA level increased intensively at first for a short period of time but after reaching a maximum value it decreased sharply, and at 50 h it reached a surprisingly low value (30 mmol/kg protein), which did not change at all at any later fermentation times. Concomitantly with these changes a decrease in the GSH concentration and an increase in the specific GST and γ GT activities were detected. Silibinin, CDNB and the mixture of borate and L-serine, which were added to the fermentation media at 30 h, all increased the intracellular POA concentrations. These results suggested that POA was eliminated in a GSH-dependent detoxification process from *P. chrysogenum* mycelia. This pathway may have a multiple physiological function in addition to the detoxification of POA regarding that it may also contribute to the formation of phenoxyacetyl-CoA [2] and to the decrease of the intracellular GSH concentration. GSH is a well-known inhibitor of the penicillin synthesis [3, 4].

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E. FODOR, E. NAGY

Karyotyping of human pathogenic *Candida* species isolated from nosocomial and recurrent infections by pulsed-field gel electrophoresis

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Candida species have emerged as an important cause of nosocomial infections, particularly when they involve immunocompromised hosts. The incidence of disseminated candidosis has also increased, as proven by large autopsy series. *Candida* species are now the fourth most commonly isolated organisms in blood cultures. Recurrent vulvovaginal candidiasis account for 20–25% of all cases of vaginitis in a variety of clinical settings. The etiology of this recurrent presence of *Candida* species in the vagina of a given patient is still unknown. Theories include frequent vaginal relapse or frequent reinfection due to exogenous acquisition of the strains. Classical biochemical tests are not satisfactory to investigate the relatedness of different *Candida* species in this respect. The technique of electrophoretic karyotyping by pulsed field gel electrophoresis (PFGE) makes it possible to separate yeast chromosomal DNA according to their size. The method can be used for the more precise differentiation of different *Candida* species and for karyotyping of the genome of yeasts, including *C. albicans*, *C. parapsilosis* (often involved in nosocomial infections) and *Torulopsis glabrata*.

In the present study different *Candida* isolates originating from the vagina of patients with recurrent vulvovaginitis and those isolated from severe infections from the PIC, surgical department and haematology department were karyotyped by PFGE methods. The results of these studies indicated a karyotyping variation among clinical isolates of *C. albicans*. We separated three different karyotypes of 27 strains of *C. albicans* and 2 of 3 *C. albicans* isolates from different sources (trachea and dialysis fluids) in one patient. Two *C. parapsilosis* strains were isolated from blood cultures of 2 patients in the same period of time. They had the same electrophoretic karyotypes and hence the common origin of these strains was proven. Women with recurrent vulvovaginal candidiasis due to *C. albicans* and *T. glabrata* were followed for a mean of 4–12 months. The results confirmed the similarity of the types of *C. albicans* and *T. glabrata* and

demonstrated the persistence of colonization with the same strain over different periods of therapy.

A. LUKÁCSI, I. PFEIFFER, J. KUCSERA

**Comparative study of *Saccharomyces dairensis*
and *Saccharomyces castellii* strains**

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Recently the reclassification of the *Saccharomyces dairensis* CBS 4309 strain into a new species (*Saccharomyces castellii*) raised problems in the genus *Saccharomyces*. It became necessary to reconsider the proper taxonomic place of the strains registered as *S. dairensis*.

During our study we have checked the reliability of classical (cycloheximide sensitivity, carbon assimilation spectra) and molecular (RAPD and *Hae*III-digested pattern of the total DNA, isoenzyme analysis, RFLP of the mitochondrial DNA, electrophoretic karyotype) taxonomic methods for this purpose. Besides the comparative study of the type strain of *S. castellii* (CBS 4309) and the type strain of *S. dairensis* (CBS 421) the strain *S. dairensis* CBS 1579 was also involved in the analysis.

It was established that the cycloheximide sensitivity, carbon assimilation spectra, isoenzyme pattern, the RAPD and *Hae*III pattern of the total DNA, moreover the electrophoretic karyotype are suitable methods to separate the two species. However, no difference was found in the RFLP of their mitochondrial DNA. Furthermore our results suggest that the strain *S. dairensis* CBS 1579 should be replaced into the species *S. castellii* according to its similarity to the strain *S. castellii* CBS 4309.

M. GYANTÁR, I. PFEIFFER, J. KUCSERA

**Isolation of dsRNA associated virus-like particles
from *Cryptococcus hungaricus* CBS 6569 strain**

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During our preliminary study on *Cryptococcus hungaricus* the existence of dsRNA molecules with molecular weight 1.9 and 4.6 kb was revealed in strain CBS 6569. The RNase protection assay suggested that these molecules were encapsidated in a protein coat. Further analysis verified that this dsRNAs copurified with an icosahedric virus-like particle, 37 nm in diameter.

In *S. cerevisiae* dsRNAs confer the killer phenotype of the host strain. Therefore the killer activity of *C. hungaricus* strains was tested. It was established that strain CBS 6569 reduces a proteinaceous toxin what has maximum activity on the fourth day, at low pH range like *S. cerevisiae* killer toxin. Despite the similarity of the *S. cerevisiae* and *C.*

hungaricus dsRNAs in their sizes and the activity of their toxin at the same pH range, their activity spectra were different. *C. hungaricus* toxin had effect only on strain *C. hungaricus* CBS 4214 and no effect on *S. cerevisiae* sensitive strains, moreover, *C. hungaricus* strains were not sensitive to *S. cerevisiae* toxin.

We attempted to cure *C. hungaricus* of the dsRNAs by UV irradiation, cycloheximide and ethidium bromide treatment. All of our attempts failed, therefore there is no direct evidence if the toxin production is associated with the presence of dsRNAs.

G. BUJDOSÓ, T. DEÁK

Identification of yeasts isolated from vineyards

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Yeasts collected from serial samples taken from different vineyards (Etyek, Nagyréde) were examined. The samples were taken from soil, grape leaves, rotten leaves, clasper, various mouldy and sound grapes of cultivars Muscat Ottonel, Zweigelt, Chardonay. In order to isolate variant groups of yeasts including also minor species, the following selective media were used: Yeast Carbon Base + Cellobiose, Yeast Nitrogen Base + Lysine, Plate Count Agar (PCA) + cycloheximid (0.01%) and Dichloro Rose Bengal Chloramphenicol (DRBC) Agar. The media also contained chloramphenicol (0.01%) to inhibit growth of bacteria. The preliminary identification of yeasts was accomplished by traditional methods including the assimilation of glucose, erythritol, cellobiose, mannitol, maltose, raffinose, inositol, melibiose and nitrate, the fermentation of glucose, and the urease test. The average yeast population in samples ranged from 10^4 to 10^5 per g.

A total of 232 isolates were collected, of which 36% was basidiomycetous yeasts based on positive urease tests. Most of these (80%) was red yeasts (*Rhodotorula* and related species). The most frequent ascomycetous yeasts belonged to genera *Kloeckera*, *Metschnikowia*, *Debaryomyces* and *Candida*. In the samples collected previously, no genuine *Saccharomyces* wine yeasts were found

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Physical maps of the mitochondrial DNAs of some isolates of the *Aspergillus niger* species aggregate

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High degrees of intra- and interspecific DNA polymorphisms were observed among imperfect black *Aspergillus* isolates [1, 2, 3]. Strains of the *Aspergillus niger* species aggregate could be divided into three species, *A. niger*, *A. tubingensis* and “A.

brasiliensis" based on their nuclear and mitochondrial DNA (mtDNA) RFLPs. Vegetative incompatibility represents a strong barrier in intra- and interspecific crosses; interspecific hybrids could not be established, although mitochondrial transmission and mitochondrial DNA recombination were readily observed both in intra- and interspecific relations [4]. While the structure and organisation of mtDNA of *A. nidulans* were studied in detail [5], only limited data are available for those of black *Aspergilli*: A physical map of the mtDNA of a single *A. tubingensis* strain has been reported so far [6]. In order to clarify the possible differences in the sizes and organisations of the mtDNAs of black *Aspergillus* strains, we constructed the physical maps of some strains belonging to different species of the *A. niger* species aggregate. The sizes of mtDNAs of the two examined *A. niger* strains were 31.2 and 31.7 kb, while those of an *A. tubingensis* and an "*A. brasiliensis*" strain were 37.4 and 39.6 kb, respectively. The order of the genes was found to be similar to that on the mtDNA of *A. nidulans*, differences were mainly observed in the relative positions of some genes, possibly due to the presence or absence of some intergenic sequences.

Successful mitochondrial transfers were carried out between incompatible strains possessing different mtDNA patterns belonging to the *A. niger* species aggregate. Isolates with recombined mtDNAs were readily observed. By preparing the functional maps of these recombinant mtDNAs we might have a chance to follow the recombination events in detail.

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Transmission of mitochondria and recombination of mtDNA in *Aspergillus niger* complex

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Considering the banding patterns of *Hae*III-*Bgl*II digested mitochondrial DNA (mtDNA) of the strains belonging to *Aspergillus niger* species aggregate can be divided into three main groups. The first and second groups respond to *A. niger* and *A. tubingensis* species, respectively, while the third, possibly a new species, is represented by some Brazilian isolates (the mtDNA groups 1, 2, and 3, corresponding to nuclear ribosomal RNA genes groups I, II, and III). The mtDNA 1 and 2 groups consist of several subgroups [1]. All these strains, which represent different molecular types, revealed full incompatibility in respect of nuclear complementation. Mitochondrial transfers were

performed by protoplast fusion under selection pressure, using as donor a mitochondrial oligomycin-resistant (oliR) mutant, which exhibits type 1a mtDNA and type 1 rDNA. Following protoplast fusion in the presence of oligomycin, mitochondrial oliR progenies could be recovered by selecting for the nuclear phenotypes of the oliS recipient strains. The transfers were successful in all possible combinations. Recombination of mtDNA occurred among strains representing different mtDNA groups or subgroups. Within the group of strains belonging to mtDNA type 1, the intraspecies transfers resulted in a single type of recombinant RFLP profile in each subgroup. The recombination events were more complex when the transfer of oliR occurred between strains representing different species. A great variety of recombined RFLP profiles of the partners appeared. In mtDNA transfers 1a→2b and 1a→2d, resistant clones harbouring possibly unchanged donor mitochondria with the nuclear background of the oliS recipient partner (mtDNA type 1a with rDNA type II) were recovered in addition to the recombinant types.

The resistant strains harbouring either recombinant or substituted mtDNAs were crossed with a compatible sensitive parent of isogenic origin by anastomosis. Oligomycin was not applied for selection during heterokaryon formation. Heterokaryons possessing mixed mitochondrial populations at the beginning segregated for homoplasmic sectors during prolonged cultivation. Conidia harvested from either resistant or sensitive sectors of heterokaryons resulted in both parental nucleotypes of progenies. An overwhelming majority of the progeny harboured recombined mitochondria (even the sensitive ones). This result suggests that mitochondrial recombination may also occur without any selection pressure.

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¹M. VASTAG, ¹T. PAPP, ²Á. NAGY, ¹ZS. PALÁGYI, ¹L. FERENCZY
and ¹CS. VÁGVÖLGYI

Delimitation of *Rhizomucor* species on the basis of genetic and physiological markers

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The genus *Rhizomucor* comprises three species: *R. pusillus*, *R. miehei* and *R. tauricus*. Whereas *R. tauricus* has only a single known isolate, the other two *Rhizomucor* species are well represented in nature. They are important both as agents of opportunistic fungal infections and as producers of different enzymes of industrial importance. The aim of the present study was to find a new approach for the safe differentiation and identification of *R. miehei* and *R. pusillus* isolates.

Twenty-one *Rhizomucor* isolates from various sources were assayed for their ability to utilize 90 various substrates as a single carbon source. Besides saccharose, characteristic differences were found in the utilization of tryptophan, glycine, phenylalanine and β -alanine. The isolates were also examined for their isoenzyme

activities: among the five enzyme systems tested, esterase and malate dehydrogenase proved useful in affording markers of distinctive value.

These data (supplemented with the results of morphological and mating studies) were used for numerical analyses to obtain preliminary information on the genetic polymorphisms of the *Rhizomucor* species. Higher variability was found among the heterothallic *R. pusillus* isolates, while the group of *R. miehei* isolates was found to be the more homogeneous genetically.

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A. KÓNYA, A. JEKKEK, J. SÜTŐ, J. SALÁT

Optimization of compactin fermentation

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Compactin was isolated first from fermentation broth of *Penicillium brevicompactum* in 1976 as an antifungal metabolite, and its hypocholesterolemic activity was published also in 1976. Compactin is the precursor of pravastatin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase, applied in the clinical treatment of hypercholesterolemia. Pravastatin is produced by a two-step fermentation process, in which the first step is the production of compactin by a fungal strain and the second one is the microbial hydroxylation of compactin.

A compactin producing *Penicillium sp.* strain was isolated from a soil sample in our institute. The compactin yield of *Penicillium sp.* IDR-629 was improved from 5 µg/ml to 250 µg/ml by mutation-selection methods. A further enhancement of compactin productivity was promoted by medium optimization. We have found that the most effective carbon source is glucose and the most effective nitrogen source is yeast extract. Magnesium-sulphate in the production medium increases the compactin yield, too. It is difficult to investigate the quantitative effects of three factors in a single experiment. So we used non-linear regression analysis with Box-Wilson central composite design experiments and the steepest ascent method to determine the optimal concentration of the components in the production medium. In the first experiment we determined the regression coefficients and the gradient vector, which determines the path to the optimal concentrations. A second regression experiment was done in the near of the optimum to calculate the correct concentrations of the ingredients. The fitted equations were examined with F-test. With this method the compactin productivity was increased to 390 µg/ml.

AGRICULTURAL AND FOOD MICROBIOLOGY

K. KLÓSZ, I. KISS, J. FARKAS

Determination of efficiency of disinfectants by bioluminometry

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The microbiological cleanliness of working surfaces and technological lines can be determined by various, rapid methods. Among these the bioluminescence measurements should be mentioned which can be carried out with sufficient accuracy on producing lines – i.e. in place – within 10 minutes.

In model experiments the bactericide efficiency of food disinfectants and detergents was studied by ATP bioluminometry. The survival of certain strains of *E. coli* and *Micrococcus* sp. has been investigated in the function of concentration and treatment time of HYPO, SANOSIL and DIVOSCHAUM. The measurement was performed with a BIO-ORBIT LUMINOMETER.

The killing effect of detergents and disinfectants can be followed well increasing the concentration and treatment time. The number of surviving bacteria was proportional with the quantity of ATP content in their cells. It could be proved that integrated value of light intensity vs. time during the luminometric test showed a strong correlation with the results of colony counting methods (plate counting and the spiral-plate technique, respectively). The efficiency of disinfectants and the disinfection treatment can be very quickly determined and monitored with this bioluminescent method.

¹L. VOLODYMYR, ¹P. VOLODYMYR, ²B. NADYA

Comparative evaluation of designed and developing probiotal biopreparation effectiveness

¹ National Agrarian University, Kyiv, ² Uzhhorod State University, Ukraine

Experiments were performed on laboratory animals (outbred white mice) with artificial medicinal disbacteriosis caused by tetracycline injectioning (50 mg/g during 5 days). Disbiotic changes were characterized by the considerable decrease of obligate microflora (*Lactobacillus* and *Bifidobacterium*) and significant increasing of *Escherichia*, *Proteus*, *Enterococcus*.

To normalize microbiocenosis Sporolact, SL-bacterine, Lactobacterine, Monosporine-PK were injected to mice of different groups. The result of the study show that after the application of the complex sporolact preparation, regeneration of the quantity of bifidobacteria up to the standard indices, and decrease (also to the standard

indices) of the quantity of *Proteus*, *Staphylococcus*, yeast-like fungi were observed. Upon discontinuance of tetracycline injection and prior to Sporolact and Monosporine-PK treatment practically no bifido- or lactobacteria were revealed, whereas on the 6th day after accomplishment of the treatment, lactobacteria were noticed to be plating in amount of $2.7 \times 10^7 - 5.4 \times 10^7$ CFU/g, bifidobacteria – 10^8 CFU/g. Simultaneously, staphylococci content decreased more than 100 fold, whilst that of *Proteus* or *Candida* genera microbes – no less than 10 fold.

Application of SI-bacterine in the same doses and following the same scheme resulted in the content decrease of the most potentially pathogenic microorganisms down to the standard indices. Although, quantitative regeneration of obligate representatives of normoflora, e.g., of lactobacteria was proven less intensive. When Lactobacterine was injected to the animals, regeneration of bacterial content of lactobacteria up to $10^7 - 10^8$ was registered, whereas simultaneous oppression of *Proteus* genera microbes, yeast-like fungi and other potentially pathogenic microorganisms was less obvious.

Maximum effectiveness was elicited by Sporolact and Monosporine-PK.

D. PRIBÉK and R. GÁBORJÁNYI

Different serotypes in Hungarian plum pox isolates

Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary

Fifteen representative isolates were selected from more than hundred samples of plum pox virus (PPV) according to their serological reactions in agar-gel diffusion tests. Isolates were inseparable on the basis of symptoms on test plants. Using monoclonal antibodies raised against different (external, intermediate and internal) epitopes of PPV in indirect ELISA (IDAS) the presence of both M and D serotypes has been demonstrated, indicating that both types common in Hungarian orchards. Some isolates showed intermediate characteristics between the two main serotypes.

¹Z. KISS, ²M. KECSKÉS

Tricalcium-phosphate mobilization by soil- and rhizosphere bacteria

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Phosphorus plays a key role in energy transformations and it is essential for the growth and development of microorganisms, plants and animals. Microbes have an important role of plants phosphorus supply.

Phosphate-solubilizing ability of different rhizosphere (e.g. gerbera, black locust, chickpea) bacteria and 17 *Bacillus* strains (3- *B. insolitus*, 4- *B. macerans*, 2- *B. megaterium*, 1-1 *B. azotoformans*, *B. laevolacticus*, *B. firmus*, *B. polymyxa*, *B. psychrophilus*, *B. subtilis*, *B. cereus*, *B. thuringiensis*) were tested on tricalcium-phosphate containing Pikovskaya media/broth. The determination of organic acids in culture solutions [*B. azotoformans* (Z4) and *B. macerans* (Z 16)] was carried out with HPLC.

The *Bacillus* strains solubilized significantly different amount of tricalcium-phosphate even at the first day. Quantity of the solubilized phosphate of solution increased with the incubation time. *B. azotoformans* (Z4) and *B. macerans* (Z 16) were found to be the most active: at the first day they mobilized 8–10 times more phosphate than the others.

The pH of the solution decreased considerably after 24 hours. pH of the broth was dropped most strongly (from 6.7 to 4.5) by Z4 and Z16 strains. It is obvious that there is a clear correlation between the decrease of pH and phosphate-mobilization ($r = -0.924$).

With HPLC measurement it was proved that the two best phosphate-solubilizing strains produce a substance, which has not been identified yet, and lacked from the control. The amount of this substance correlated nearly ($r = 0.97$) with the amount of solubilized phosphate.

J. BITSKEY, E. K. NOVÁK, A. FÁNCZI

Microbial denitrification of drinking water

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Our task was to develop an efficient technology to eliminate a ca. 100 ppm (an average level of pollution in Hungary) nitrate from contaminated water, as the hygienic limit for drinking water in our country is at 40 ppm for adults, while at 20 ppm for babies. For the realisation of our aim various sizes of continuous flow-through reactor systems were built and was filled with different cellulose containing wastes to serve as both physical support and chemical substrate for the bacterial population developed preliminary on wheat straw, corn stems or newspaper substrates. In the first period trace elements and phosphate supplements were added, and artificial nitrate contaminated water was used. After determining the working parameters, a pilot plant system was planted on a natural nitrate contaminated aquifer. Besides the nitrate, the pH, and the levels of nitrite, ammonia, phosphate as well as the chemical oxygen demand (COD) were measured both in inflow and outflow water with different flow-rates and supplements. The best results – reduction of the nitrate content in the effluent below 10 ppm, parallel to low ammonia and nitrite (below 0.1 ppm) level, while that of COD near to the influx level or at least below 10 ppm – were obtained with 0.2 m/h flow-rate and addition of 79 ppm phosphate as well as trace elements and bios source. While to conduct the overall process towards the assimilatory nitrate reduction proved to be essential. Although the nitrite, ammonia and COD level may increase because of the bacterial activity (especially when assimilatory

nitrate reduction takes place), these secondary contaminants can easily be eliminated by a common post-reactor water treatment (purification). Based on the biological, chemical and hydrological experiences an increase of the flow rate to 1.2 m/h became possible. For potable water discarded purified cellulose was used.

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**Species composition of trichoderma community
in a rendzina soil affected by microclimatic conditions**

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To assess the influences of microclimatic conditions (namely the exposure to the sunshine) on species composition of *Trichoderma* in soil samples were collected from sides of the four cardinal points of a dolina (funnel-like surface depression in Bükk-mountain). All colonies developed from soil particles on *Trichoderma*-selective medium were identified through macro- and microscopic observations. More than 200 isolates were obtained which belonged to 10 *Trichoderma* species; in following decreasing order of relative frequency: *T. atroviride* (39.89%), *T. koningii* (37.76%), *T. pubescens* (11.17%), *T. viride* (2.66%), *T. fertile* (2.12%), *T. harzianum* (2.12%), *T. strictipilis* (1.59%), *T. hamatum* (1.06%), *T. polysporum* (1.06%), *T. spirale* (0.53%). Significant differences were found in species composition of soil samples from differently exposed sides of dolina. Only 2 species were found at southern whereas 6 species at western exposure. Shannon diversity indexes were similar: lowest (0.1917) at southern and highest (0.6107) at western exposure. *T. koningii* occurred most frequently (83.33%) at warm southern and at a lower rate (13.43%) at cold northern exposure whereas *T. atroviridae* showed a reversed tendency with highest (74.63%) rate at northern and lowest at warmer western (5.0%) exposure. It was stated that minute differences in environmental conditions resulted in marked difference in species diversity of *Trichoderma*.

G. KISKÓ

**Attempts to determine mouldiness of paprika powder
by near infrared spectroscopy**

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and Food Industry, Budapest, Hungary

Different methods are available for detecting moulds in food. They include cultural methods, detection of heat-stable enzymes or other heat-stable mould components like chitinase, microscopic methods for detecting mycelium, immunological and chemical

methods etc. Most of the current methods have disadvantages. In this work the ergosterol assay was chosen as reference method for determination the mould content in the samples because ergosterol is a mould specific component and it can be satisfactorily determined.

The task of our investigation was to develop a rapid method to detect both viable and dead mould biomasses in spice paprika powder. We tried to explore the relationship between the mould content of the paprika samples and their NIR spectra. Both quantitative and qualitative methods were used for this purpose. At quantitative investigation models were determined in the whole wavelength range. At qualitative investigation PQS (Polar Qualification System) was used described by Kaffka and Gyarmati (1991) and the "quality points" of the samples were determined using different spectral ranges. The location of the quality points in the "quality plane" clearly shows the shift of the quality points in the function of the mould content.

The NIR technique proved to be very promising as a rapid, non-destructive, reagentless method to detect the mould biomass content in paprika powder and there is simple means for monitoring the mycological quality assurance of paprika powder products.

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**Single and mixed culture growth of *Listeria*
and *Lactobacillus* strains at different environmental
conditions**

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³ BFE, Institute of Hygiene and Toxicologie, Karlsruhe, Germany

The growth of two *Listeria* and two *Lactobacillus* strains was examined in CASO broth with automatic turbidimetric system for single cultures and traditional selective plate counting method for mixed cultures. Single cultures of *Listeria innocua* and *Lactobacillus plantarum* were grown at different temperatures and initial pH (installed by lactic acid). Mixed cultures of the two strains were grown at 30 °C with varied initial cell-counts of *Lb. plantarum*. The growth of *Listeria monocytogenes* and *Lactobacillus casei* was measured at cheese ripening conditions: 13 °C, pH 5.3; 5.6; 5.9; with 0.7%, 1%, 1.3% of lactic acid. Mixed cultures of the latters were incubated in similar conditions.

Results showed that the pH-sensitivity of *L. innocua* and *Lb. plantarum* depended on the incubation temperature.

Regarding the growth of *L. monocytogenes* and *Lb. casei* at 13 °C an interaction was observed between the inhibitory effect of pH and lactic acid concentration. Lactic acid enhanced the effect of low pH.

In mixed cultures, lactic acid bacteria had an antagonistic effect on the growth of the *Listeria* species.

¹K. POSTA, ²G. FÜLEKY**Mineralization of organic phosphorus by vesicular-arbuscular mycorrhizal fungi (*Glomus mosseae*)**

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The potential of the mycorrhizal fungus, *Glomus mosseae*, to mineralize organic phosphorus was studied in pots with two compartments, one for root growth (rhizosphere) and one for hyphal growth (hyphosphere). Compartmentation was accomplished by 30 µm nylon net. The treatments consisted of sterilized soil, supply of organic (RNS, ATP) and inorganic [Ca(H₂PO₄)₂] phosphorus in the root or hyphae or in both compartments. Plants were inoculated with *Glomus mosseae* or remained uninfected.

In the rhizosphere and hyphosphere of mycorrhizal plants acid phosphatase activity was higher than by non-mycorrhizal plants. Through the hyphal compartment, phosphatase activities were distinctly higher in the presence of mycorrhizal plants particularly with supply of organic phosphorus which also increased hyphal growth length.

Phosphatase activity was strongly correlated with hyphal length. Mycorrhizal inoculation increased plant dry weight, and total P uptake irrespectively of phosphorus sources. Of the total P uptake mycorrhizal contribution accounted for 29% with P supplied in inorganic form, and 38–58% with P supplied in organic forms.

The results demonstrate a stimulatory effect of organic phosphorus on hyphal growth as well as the efficient use of RNA and ATP by phosphatase of mycorrhizal hyphae.

¹S. FARKAS, ²GY. GUNICS, ³A. HEGEDŰS, ⁴M. KECSKÉS**Effect of cadmium, selenium and silver ions on *Escherichia coli* and *Rhizobium* strains**

¹ Agricultural College of Debrecen Agricultural University, Hódmezővásárhely, ² College of Albert Szent-Györgyi Medical University, Szeged, ³ Teacher's Training College Juhász Gyula, University, Szeged, ⁴ Environmental Microbiological Research Group of the Hungarian Academy of Sciences, Gödöllő, University of Sciences, Budapest, Hungary

The water-soluble inorganic salts of metals are toxic at low concentrations too, on bacteria. We examined antibacterial effect of Cd, Se and Ag ions containing inorganic compounds on *Escherichia coli*, *Rhizobium meliloti* and *R. leguminosarum* strains in vitro.

The bacteria strains were variable sensitive to Cd ions. The minimal inhibitory concentrations (MICs) of Cd ions on *R. meliloti* and *R. leguminosarum* were at 8–32 µg/ml. The MICs of Cd ions on *E. coli* strains were at 65–256 µg/ml.

The *E. coli* strains were medium resistant to Se ions (MIC = 512–1024 µg/ml). The *R. meliloti* and *R. leguminosarum* strains were found to have high level resistance to Se ions (MIC > 2000 µg/ml).

The *R. meliloti* and *R. leguminosarum* strains were more sensitive against toxic effect of Ag ions (MIC = 0.5–8 µg/ml) than *E. coli* strains (MIC = 4–16 µg/ml).

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